

10/525256256

Rec'd PCT/PTO 22 FEB 2005

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
11 March 2004 (11.03.2004)

PCT

(10) International Publication Number  
**WO 2004/019900 A1**

- (51) International Patent Classification<sup>7</sup>: **A61K 7/48, 31/00**
- (21) International Application Number:  
PCT/EP2003/009496
- (22) International Filing Date: 27 August 2003 (27.08.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
02019244.9 27 August 2002 (27.08.2002) EP
- (71) Applicant (*for all designated States except US*): **SOCI-  
ETE DES PRODUITS NESTLE** [CH/CH]; P.O. Box 353,  
CH-1800 Vevey (CH).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): **MOODYCLIFFE,**  
Angus [GB/CH]; Avenue des Roses 4, CH-1009 Pully  
(CH). **POQUET,** Laure [FR/CH]; Route d'Amier, 16,  
CH-1092 Belmont-Sur-Lausanne (CH).
- (74) Agent: **STRAUS, Alexander;** Becker, Kurig, Straus,  
Bavariastrasse 7, 80336 München (DE).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC,  
SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA,  
UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,  
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO,  
SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM,  
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

## Published:

— with international search report

*For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.*

(54) Title: PREVENTING OR TREATING EPITHELIAL TISSUE DAMAGE OR HAIR LOSS

(57) Abstract: The present invention pertains to a method for preventing and/or treating epithelial tissue damage, such as is effected by inflammatory reactions, ageing or cancer and/or to prevent and/or treat hair loss. In particular, the present invention relates to substances and/or compositions modifying, in particular blocking endogenous CD<sub>14</sub> function. According to another aspect the present invention also provides a method for screening for compounds suitable for use in the method and the composition of the present invention.

BEST AVAILABLE COPY

WO 2004/019900 A1

### Preventing or treating epithelial tissue damage or hair loss

5 The present invention pertains to a method for preventing and/or treating epithelial tissue damage, such as is effected by inflammatory reactions, ageing or cancer and/or to prevent and/or treat hair loss. In particular, the present invention relates to substances and/or compositions modifying, in particular blocking endogenous CD<sub>1d</sub> function. According to another aspect the present invention also provides a method for screening for compounds  
10 suitable for use in the method and the composition of the present invention.

The most prominent epithelial tissue in living beings is the skin, which represents the largest organ in the organism. The system of skin integument, which comprises the epidermis, dermis and the stratum corneum, correlates with those of internal organs and concurrently  
15 interacts with the surroundings. Being the interface between the environment and organism itself, the skin is heavily influenced by external factors and also variable parameters of the organism's inner system. The skin's regulative mechanisms need, therefore, always be active to induce systemic changes necessary to maintain normal pathological events concerning skin integument morphology and activities. A great deal of processes assuring the adequate consumption of increased affluence of energetic and plastic substances according to the skin's  
20 needs become guarantors of morphological and functional stability of skin structures. So, the state of integuments determines the realization of metabolic processes necessary for skin cell viability and activity leading to the presence of healthy skin peculiarities such as barrier function, elasticity, turgor properties, humidity, pigmentation etc..

25

During the lifetime of a living being different signs, characteristic of ageing, appear on the skin, with the principal clinical signs being the appearance of fine lines and deep wrinkles which increase or are accentuated with age. Moreover, the skin's complexion is generally modified and diffuse irritations and occasionally telangiectasias may come into existence on  
30 certain areas.

These signs of ageing are even promoted by exposure of the skin to exogenous influences, such as e.g. UV-radiation, pollutants, free radicals or chemical substances.

- 5 Moderate UV exposure generally causes the well known effects of reddening the skin with an accompanying inflammation reaction, known as erythema. This phenomenon, often referred to as "sunburn", is painful and commonly results in a subsequent peeling of the skin.

Moreover, excessive UV-exposure of the skin may also lead to the onset of severe disorders, such as carcinogenesis, the most common tumours being the basal cell carcinoma (BCC), followed by squamous cell carcinoma (SCC), and more rarely malignant melanoma. Apart from damages on the DNA-level also immuno-suppression caused by UV exposure seems to account for both, non-melanoma and melanoma cancer promotion. It is presently acknowledged that photo-induced immuno-suppression permits the initiated tumour cell to evade recognition and rejection by normal immunological mechanisms, to remain latent for extended periods, and to eventually proliferate into a tumour. This concept concurs with the findings that immuno-compromised patients, whether genetically (xeroderma pigmentosum) or pharmacologically, such as e.g. organ transplant recipients, have a higher incidence of skin cancer as compared to people with a properly functioning immune system.

20

In the art several means have been proposed to prevent destructive effects of environmental factors on epithelial cells, in particular skin epithelial cells.

As regards protection to sun radiation "sun blocks" or "sunscreens" have been made available, which are applied to the skin prior to sun exposure. Typically, sunscreen compositions contain chemical agents, such as certain benzophenones, dibenzylmethanes or substituted para-aminobenzoates, i.e. compounds absorbing ultraviolet radiation, so that it cannot penetrate the skin. However, some of the compounds used for this purpose have shown to lack sufficient light stability and may even become toxic over long term application. In addition, they must stay continuously on the surface of the skin at the time of exposure to be effective. However, sunscreens are easily rubbed off or washed off by

sweating or swimming and can also be lost by penetration into the skin.

Another means to prevent skin deterioration or ageing, respectively, is to provide compounds scavenging free radicals. In this respect EP 0 761 214 discloses singlet oxygen quenchers comprising aniline derivatives and difurfuryl amine derivatives, which are reported to reduce the oxidative stress to the skin.

Yet, all these means and methods are not sufficiently capable to protect the skin from the growing challenge in our environment. To this contributes an increased atmospheric pollution and also social behaviour, according to which sun-tan is associated with health, beauty and status. As a consequence many people expose their skin to sun radiation to acquire a tan in spite of the negative results accompanying such behaviour being well known. This problem even gets more prominent with the ozone shield covering the earth becoming thinner, resulting in a heavier exposure of living beings to UV radiation.

Consequently there is a need in the art to provide a better protection of the skin to environmental factors, such as stress or sun radiation.

Accordingly, an object of the present invention is to obviate the drawbacks of the prior art and to provide such means in order to protect the skin from unfavourable influences encountered in the environment, in particular from oxidative or chemical stress or sun radiation.

This problem has been solved by providing a substance, that is capable to essentially modify, in particular block the endogenous CD<sub>1d</sub> function in epithelial cells.

In the figures,

Fig. 1A. Wild-type mice exhibit skin damage (burning) following exposure to a single dose (86mJ/m<sup>2</sup>) of UVB radiation.

Fig. 1B. Wild-type mice exhibit skin damage (burning) following exposure to a single dose (86mJ/m<sup>2</sup>) of UVB radiation. (Close-up).

Fig. 1C. CD1d knockout mice show no obvious signs of skin damage following exposure to a single dose (86mJ/m<sup>2</sup>) of UVB radiation.

Fig. 1D. CD1d knockout mice show no obvious signs of skin damage following exposure to a single dose (86mJ/m<sup>2</sup>) of UVB radiation. (Close-up).

Fig. 2 Difference in degree of UVB-induced skin damage between wild-type (Right) and CD1d knockout (Left) mice exposed to two doses (86mJ/m<sup>2</sup>) of UVB radiation.

Fig. 2A. Damaged (lesions) dorsal skin of wild-type mice exposed to two doses (86mJ/m<sup>2</sup>) of UVB radiation (Close-up).

Fig. 2B. Undamaged dorsal skin of CD1d knockout mice exposed to two doses (86mJ/m<sup>2</sup>) of UVB radiation.

Fig.3. CD1d knockout mice exhibit increased epidermal apoptosis in their dorsal epidermis compared to wild-type mice, as measured by TUNEL. Wild-type (A) and CD1d knockout (B) mouse skin not exposed to UV-irradiation. Wild-type (C) and CD1d knockout (D) mouse skin 48h after a single exposure (86mJ/m<sup>2</sup>) to UV-B radiation.

Fig. 4 a and b are graphs indicating the approximate amount of CD1d in different tissues in mice and human.

Fig. 5 shows that CD1d protein is expressed in the epidermis of mouse skin 72h following exposure to a single dose (430mJ/cm<sup>2</sup>) of UVB radiation;

Fig. 6 shows that murine skin CD1d gene transcription is regulated following UVB irradiation;

Fig. 7 shows that murine skin CD<sub>1d</sub> gene transcription is regulated following solar simulated light irradiation;

5 Fig. 8 shows that CD<sub>1d</sub> gene transcription in immortalized (DK7) human keratinocytes is regulated following solar UV irradiation;

Fig. 9. shows that COX-2 and TNF-alpha mRNA levels are down-regulated in UVB-irradiated CD<sub>1d</sub> knockout mouse skin.

10

Figure 10 a and b) show that mouse skin IL-6 and MIP1-alpha protein levels 48h after UVB irradiation are significantly decreased in CD<sub>1d</sub> KO mice.

15 Fig. 11 shows that hydrocortisone suppresses CD<sub>1d</sub> transcription in cells exposed to a chemical stress; and

Fig. 12 shows that CD<sub>1d</sub> is expressed in human hair follicles.

20 The present invention is essentially based on the finding that CD<sub>1d</sub>, a transmembrane protein expressed by a number of different cells, in particular epithelial cells, modulates a variety of different responses of the cell to stress. As will become evident from the following detailed description of the preferred embodiments, essentially modifying, specifically blocking the endogenous CD<sub>1d</sub> function in cells bearing said membrane molecule allows to prevent the detrimental effects of stress, including ultraviolet radiation-induced skin damage, e.g. as a  
25 result of burning, epidermal hyperplasia, mutant p53 accumulation, inflammation, immune suppression and skin ageing. Even more surprising is the finding that when essentially blocking CD<sub>1d</sub> function in epithelial cells induction of cancer in said cells, i.e. basal cell carcinoma, squamous cell carcinoma, malignant melanoma, colon, breast, liver, prostate, kidney, pancreas cancer etc., may be prevented. In addition, it has been surprisingly found  
30 that modifying, in particular blocking CD<sub>1d</sub> function influences hair growth and/or development.

CD<sub>1d</sub> as such is a type 1 transmembrane MHC class 1 like protein that non-covalently associates with  $\beta_2$ -microglobulin. The CD<sub>1d</sub> molecule is recognized by a T-cell receptor of natural killer T-cells (NKT) which play a role in immune modulatory and effector reactions.

5 It has been demonstrated that CD<sub>1d</sub> may present lipids to NKT cells for their activation, which notion is supported by the CD<sub>1d</sub> crystal structure having two highly hydrophobic grooves, necessary for presenting hydrophobic molecules such as lipids to the immune system.

10 In the studies leading to the present invention it has surprisingly been noted that CD<sub>1d</sub> gene transcription in mouse skin is responsive to external stress, such as UV radiation, which finding has been confirmed in human keratinocytes. In addition it has been noted that skin CD<sub>1d</sub> mediates UV-induced skin damage/inflammation by inducing COX-2 and TNF- $\alpha$  gene transcription and also inhibiting UV-induced apoptosis.

15

Without wishing to be bound to any theory it is currently assumed that one of the endogenous tasks of CD<sub>1d</sub> in living organisms is to directly control normal epithelial cell homeostasis. Normal skin homeostasis is dependent on the critical and fine tuned balance between epidermal differentiation, apoptosis, proliferation and anti-apoptosis of epidermal cells. In the  
20 skin, these processes are regulated via lipids, in particular by means of ceramides and glucosylceramides (sphingolipids). While the nucleated cell layers generate glucosylceramides (GlcCer), the proportions of GlcCer to Cer decrease late in epidermal differentiation, with the Cer content peaking in the stratum corneum acting as extracellular constituents of the epidermal permeability barrier. In addition to their structural properties, ceramides are  
25 associated with inhibition of cellular proliferation, induction of cellular differentiation and programmed cell death. In contrast, GlcCer induce cell proliferation and inhibit programmed cell death.

Based on the findings in the present invention, CD<sub>1d</sub> appears to be one of the receptors via  
30 which the above mentioned lipids might fulfil their biological task. Specifically, CD<sub>1d</sub> seems to negatively regulate apoptosis. In consequence, in cells under a stress situation, e.g. when

exposed to UV-radiation, CD<sub>1d</sub> supports a continued existence of said stressed cells, even when their genetic material is damaged and/or mutated, which damaged cells will contribute to inflammation processes induced and eventually account for the phenomenon of ageing or eventually tumour development.

5

In blocking and/or modifying endogenous CD<sub>1d</sub> function, apoptosis of cells under stress may be promoted, instead of their survival and propagation, with the effect that cells that have been damaged to a certain extent, particularly at the DNA level, do not have the chance to proliferate and in case disseminate in the body. The cells once dead will then be extinguished by natural processes in the body and be replaced by "healthy" epithelial cells. Likewise, by means of blocking or modifying CD<sub>1d</sub> also an interaction with NKT is substantially prevented or altered, wherein the phenomenon of immune suppression during exposure to UV radiation will be essentially reduced or barred at all. Also, this condition is supposed to assist the organism's immune system to eradicate damaged cells, brought about by exposure to UV.

15

The substance capable of blocking and/or modifying the CD<sub>1d</sub> transmembrane molecule's activity may be any substance interfering with the endogenous biological function of CD<sub>1d</sub>, and in particular preventing or reducing association of CD<sub>1d</sub> with endogenous or exogenous lipids. The substances are obtainable by a process comprising the steps of (a) exposing epithelial cells to a substance of interest, (b) subjecting the epithelial cells to a stress situation, (c) determining the effect of said stress to said epithelial cells by screening for one or more of the following assays: (i) epithelial hyperplasia (H&E), (ii) epithelial proliferation (BrUd, PCNA), (iii) epithelial apoptosis, (iv) p53 mutation accumulation, (v) quantitative and qualitative assessment of epithelial lipids, (vi) co-clustering patterns of apoptotic and non-apoptotic cell surface receptors, (vii) production of pro-inflammatory cytokines, (viii) production of immuno-modulatory cytokines, (ix) markers of inflammation, (x) anti-apoptotic transcription factor activity (xi) markers of ageing, and (d) comparing the results obtained with a control. Such a control may e.g. be an assay, wherein the cells have been subjected to the same stress situation, wherein, however, no substance to be investigated had been added (negative control). Likewise a control may also be, including a substance with a known positive effect in the assay and determining the difference in effect achieved by the

30



substance investigated and the known substance (positive control).

A substance is considered to be active in the context of this application, in case it prevents the negative effects of stress as detailed according to any of the above assays.

5

It will be appreciated that CD<sub>1d</sub> activity may be blocked and/or modified by substances acting on the genetic level or at the protein level.

10 Substances acting on the genetic level are compounds influencing, in particular preventing transcription or translation of the CD<sub>1d</sub> gene, such as polynucleotides anti-sense to at least a part of the CD<sub>1d</sub> gene or the CD<sub>1d</sub>-mRNA.

15 The terms oligonucleotide and polynucleotide, which are interchangeably used herein, include linear oligomers/polymers of natural or modified monomers or linkages, including desoxyribonucleosides, ribonucleosides,  $\alpha$ -anomeric forms thereof, polyamide nucleic acids, and the like, capable of specifically binding to the target nucleic acid by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Usually the monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size  
20 from a few monomeric units, e.g. 3-5, to several 100 or even thousands of monomeric units.

25 The (anti-)sense oligo-/polynucleotides may also contain pendent groups or moieties, to enhance specificity, nuclease resistance, delivery, or other property related to efficacy, such as e.g. cholesterol moieties, duplex intercalators such as acridine, poly-L-lysine, "end capping" with one or more nuclease-resistant linkage groups such as phosphorothioate, and the like. The corresponding oligonucleotide may be used for blocking transcription, RNA processing and/or translation of the mRNA. Consequently, the oligonucleotide may comprise exon, but also intron sequences of the CD<sub>1d</sub>-target gene, as desired.

30 The nucleotide sequence of the human CD<sub>1d</sub> gene or mRNA is obtainable from NCBI (Accession numbers: AP002532 and NM\_001766, respectively). Based on his general

knowledge and skill, the skilled person may select at least a portion of the coding region of the CD<sub>1d</sub> gene and design an appropriate anti-sense polynucleotide, that prevents transcription and/or translation of the CD<sub>1d</sub> gene. Likewise, also a part of the non-coding region of the CD<sub>1d</sub> gene may serve as an agent for preventing transcription or reducing the number of transcripts, respectively, of the CD<sub>1d</sub> gene. Here, in particular parts of the promoter region may serve as a template for preparing an antisense polynucleotide, but likewise transitions regions from introns and exons and vice versa. According to a preferred embodiment such a substance may be an DNA or a cRNA (RNA-interference).

- Yet, apart from the CD<sub>1d</sub> gene being the target, also the activity of a number of regulatory molecules which control epithelial homeostasis such as ceramides and/or glucosylceramides, may be modified such, that they exert the desired effect on the CD<sub>1d</sub> molecule. To this end, the number of the glucosylceramide synthase transcripts may be reduced by designing an polynucleotide antisense to at least a part of the glucosylceramide synthase gene or glucosylceramide synthase mRNA, so that eventually the signal to epithelial cells to proliferate is turned down. The nucleotide sequence of the glucosylceramide synthase gene is disclosed in Ichikawa et al., PNAS 93 (1996), 4638-4643, which document is incorporated herein by way of reference. Likewise, non coding regions may serve as a template for the antisense polynucleotide, such as the promoter region and/or transitions from introns to exons and vice versa. According to a preferred embodiment such a substance may be a DNA or a cRNA (RNA-interference).

Apart from reducing the proliferation signal also the signal driving epithelial cells to apoptosis via the CD<sub>1d</sub> molecule may be enhanced. In this respect the number of corresponding transcripts may be increased, which may be effected by providing a higher number of polynucleotides encoding a sequence comprised by the sphingomyelinase or ceramide synthase gene and/or the sphingomyelinase or ceramide synthase mRNA.

Apart from the genetic level, the biological activity of the CD<sub>1d</sub> molecule may also be modified, in particular blocked at the protein level, in particular by any substance binding to the CD<sub>1d</sub> receptor on or in epithelial cells and blocking the endogenous biological functionality thereof.

According to a preferred embodiment the substance capable of modifying, in particular blocking biological CD<sub>1d</sub> function is a polypeptide or a peptide, in particular hydrophobic peptides, more preferably an antibody, or a part thereof, that binds to the CD<sub>1d</sub> receptor and  
5 blocks its biological function, such as the interaction with NKT. As parts thereof, in particular mini-antibodies are envisaged lacking the F<sub>c</sub>-part. According to an alternative embodiment the substance capable of blocking the biological CD<sub>1d</sub> function may also be a soluble CD<sub>1d</sub> receptor, that is, that part of the polypeptide lacking the region, anchoring the polypeptide in the membrane. The soluble CD<sub>1d</sub> receptor will scavenge the in vivo ligands  
10 that promote survival of the stressed cells, thus promoting apoptosis. In addition, binding of the natural killer cells to CD<sub>1d</sub> in vivo will be reduced, thus preventing activation of the T-cells and consequently inflammatory and/or immunosuppressive reactions.

According to a preferred embodiment the substance capable of blocking and/or modifying  
15 biological CD<sub>1d</sub> function is a lipid derived from a plant, microbe or animal, including a phospholipid, ganglioside, sphingolipid, glycosphingolipid, phosphatidylinositol phosphate, sterol, polyphenol, glyceride or fatty acid. These lipids may influence CD<sub>1d</sub> function by directly binding the CD<sub>1d</sub> molecule or indirectly by influencing CD<sub>1d</sub> gene expression.

20 According to an alternative embodiment the substance capable of blocking and/or modifying biological CD<sub>1d</sub> function is a ceramide, such as ceramide 8 or sphingosine phosphocholine or a ligand of a receptor belonging to the TNF-superfamily, in particular CD95/APO-1/Fas, which induces apoptosis thus interfering with the anti-apoptotic function of CD<sub>1d</sub>. In another embodiment the objective substance is an organic compound obtained by chemical synthesis.

25

It is well established that ceramide glycosylation, via glucosylceramide synthase, and the subsequent build up of glucosylceramides allows cellular escape from stress-induced programmed cell death, conferring cancer cell resistance of a variety of cancers including breast, skin, colon and epitheliod carcinomas, to cytotoxic anti-cancer agents. As CD<sub>1d</sub> can  
30 bind glucosylceramide and is over-expressed by the same multi-drug-resistant cancer cells (e.g squamous cell carcinoma), it is envisioned that the anti-apoptotic activity of CD<sub>1d</sub>

regulates cancer cell resistance to cytotoxic drugs, possibly at the level of protein-glucosyl-ceramide binding. Thus, in principle the substances of the present invention that block and/or modify endogenous CD<sub>1d</sub> function strongly decrease multi-drug resistance of a variety of cancers including skin, gut and breast cancers.

5

In principle, the substances of the present invention may also influence the bi-directional trafficking of CD<sub>1d</sub> to and from the membrane.

The substances may be included in any composition suitable for administering the substance to an individual, in particular a food composition, a cosmetic composition or a pharmaceutical composition.

10

The pharmaceutical compositions containing at least one of the substances capable of blocking or modifying the CD<sub>1d</sub> surface molecule according to the invention can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described herein under, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "a therapeutically effective dose". Amounts effective for this will depend on the severity of the disease and the weight and general state of the patient.

15

20

In prophylactic applications, compositions containing at least one of the substances capable of blocking or modifying the CD<sub>1d</sub> surface molecule according to the invention are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be "a prophylactic effective dose". In this use, the precise amounts again depend on the patient's state of health and weight.

25

The compounds of the invention are preferably administered with a pharmaceutical acceptable carrier, the nature of the carrier differing with the mode of administration, for example parenteral, intravenous, oral and topical (including ophthalmic) routes.

30

The desired formulation can be made using a variety of excipients including, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin cellulose, magnesium carbonate. This composition may be a tablet, a capsule, a pill, a solution, a suspension, a syrup, a dried oral supplement, a wet oral supplement, dry tube-feeding, wet tube-feeding etc.. In order to control the drug release, sustained-release formulations can also be used.

The kind of the carrier/excipient and the amount thereof will depend on the nature of the substance and the mode of drug delivery and/or administration contemplated. E.g., for formulations containing weakly soluble antisense oligonucleotides, micro-emulsions may be employed, for example by using a non-ionic surfactant such as Tween 80 in an amount of about 0.04-0.05% (w/v), to increase solubility. Other components may include antioxidants, such as ascorbic acid, hydrophilic polymers, such as, monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, dextrans, chelating agents, such as EDTA, and like components well known to those in the pharmaceutical sciences. These various components utilized provide a variety of functions, including regulation of drug concentration, regulation of solubility, chemical stabilization, regulation of viscosity, absorption enhancement, regulation of pH, and the like. For example, in water soluble formulations the pharmaceutical composition preferably includes a buffer such as a phosphate buffer, or other organic acid salts, preferably at a pH of between about 7 and 8.

It will be appreciated that the skilled person will, based on his own knowledge select the appropriate components and galenic form to target the active compound to the tissue of interest, e.g. the colon, stomach, skin, kidney or liver, taking into account the route of administration which may be by way of injection, topical application, intranasal administration, administration by implanted or transdermal sustained release systems, and the like.

The objective substance may also be formulated in a cosmetic product, such as lotions, shampoos, creams, sun-screens, after-sun creams, sun-blocker, anti-ageing creams, ointments and/or anti-hair loss liquids. This proves in particular advantageous for essentially blocking

CD<sub>1d</sub> function in the skin and to prevent the adverse effect of sun radiation, photo-ageing and exposure of the skin to free radicals. Thus, e.g. by providing a sun-screen containing in addition to a common agent, absorbing UV-light a substance as defined herein, a protection to the sun may be provided, which by far exceeds anything known so far. This feature is based in particular on the fact that the objective substance will penetrate the skin and exert its effect after having reached the target molecules. Since this effect will stay for a while, protection to the sun will even be present in case the sun-screen has been rubbed off or has been washed off, as e.g. during sport etc. Yet, apart from sun-screens the objective substances may be included in common day-creams, lotions etc. to prevent negative effects of the daily environment, including pollution, oxidative stress etc.. It will be appreciated that the present cosmetic products will contain a mixture of different ingredients known to the skilled person, ensuring a fast penetration of the objective substance into the skin and preventing degradation thereof during storage.

Another high important composition according to the present invention is food material. In our present society a great deal of food is ingested, such as sausages, salted or grilled meat etc., that contains preservatives, ingredients or substances, that are injurious to the gut. E.g. grilled meat contains aliphatic and aromatic compounds known to be cancerogenic. Also preservatives, that kill micro-organisms contained in food material (e.g. sausages) by manipulating their DNA, will exert a similar effect to cells of the gut. In fact, the number of intestinal cancer is steadily increasing in our society, which may be attributed at least in part to the type of food taken by humans.

Consequently, the present invention provides a food composition that prevents the onset and/or development of such gut disorders, such as a composition selected from the group consisting of milk, or fermented milk products, such as e.g. yogurt, curd, cheese, milk based fermented products, ice-creams, milk based powders, infant formulae, cereal products and fermented cereal based products, mineral water, chocolate or pet food containing at least a substance capable of essentially blocking and/or modifying CD<sub>1d</sub> function. Since the objective compound will be contained in a food material in amounts, that do not affect the original taste thereof, the consumer will not notice any change in the product, but will

experience the beneficial effects thereof, namely a protective or even curing effect. Once the food material has been ingested the objective substances will arrive at the target cells, which may be epithelial cells of the gut, i.e. of the stomach or the intestine, and will bind to the CD<sub>1d</sub> receptor and exert its activity. As a consequence, cells, that are already damaged will preferably go to apoptosis instead of being maintained in said damaged form.

Since epithelial cells bearing CD<sub>1d</sub> have been found in a number of organs, such as the liver, the small intestine, the colon, the kidney, the prostate, the uterus, the pancreas, breast, skin and conjunctiva, the choice of the composition as detailed above will, by and large depend on the target tissue. As will be understood, for skin a cosmetic product might be the composition of choice, while in case of delivering the objective substance directly to the gut or the colon, a food product may be first choice. However, a food product may also be suitable for delivering the objective substance or substances to other organs, such as the kidney or the liver, which will depend on the stability of the substance in the body and its capacity of being absorbed by the body in the gut. Since food is a daily ingested material such a product offers a great variety of different possibilities. Yet, in case the objective substance is prone to degradation in the gut a pharmaceutical composition may be selected, providing e.g. encapsulation or other galenic forms to deliver the objective substance to the target tissue/to target cells.

It will be understood that the concept of the present invention may likewise be applied as an adjuvant therapy assisting in presently used medications. In this respect the pharmaceutical composition of the present invention may be administered together with e.g. cytostatika so as to prevent escape of the tumor treated from the treatment, which sometimes occurs in long term treatments of certain tumors or to assist in killing residual cancer cells not captured with the pharmaceutical regimen. Since the substance(s) of the present invention may easily be administered together with food material special clinical food may be applied containing a high amount of the objective substances. Also melanoma may be directly treated with an antibody medication against melanoma together with a pharmaceutical composition or a cosmetic product as described herein. It will be clear that on reading the present specification together with the appending claims the skilled person will envisage a variety of different

alternatives to the specific embodiments mentioned herein.

In principle, the substances according to the present invention may be used for the treatment and/ or prevention of damages in epithelial tissues, such as e.g. in the skin, gut, eye, lung,  
5 liver, prostate, breast, kidney and/or in the uterus, which are produced by a stress situation, e.g. by means of a chemical, biological or a physical stress, e.g. by exposure to oxidants or carcinogens, exposure to bacteria, viruses, fungi, lipids derived from surrounding cells and/or microbes, or exposure to UV-irradiation. Likewise, the substances may be utilized for preventing and/or treating hair loss.

10

Consequently, the substances and/or compositions according to the present invention may be utilized for treating and or preventing damages of the skin, in particular actinic and ageing damages of the skin such as dryness, actinic keratoses, irregular pigmentation (notably comprising freckling, lentigines, guttate hypomelanosis and persistent hyperpigmentation),  
15 wrinckling (notably comprising fine surface lines and deep furrows), stellate pseudoscars, elastosis, inelasticity, telangiectasia, venous lakes, purpura, comedones, sebaceous hyperplasia, acrochordon, cherry angioma, seborrhea keratosis, lentigo, basal cell carcinoma and squamous cell carcinoma, skin burning and/or blistering, cataract formation, epidermal hyperplasia, inflammation, immune suppression, and cancer, e.g. non-melanoma  
20 and melanoma skin cancers.

In order to arrive at additional substances having the above characteristics the present invention also provides a method for screening for such substances. In this method epithelial cells are utilized that may be in the form of a primary culture, i.e. directly derived from an  
25 individual or in the form of a cell line. For carrying out the method a cell culture is particularly preferred, since it allows for the continuous supply of epithelial cells during the experiments. Care must be taken that the cell culture of epithelial cells used exhibit the same phenotypic traits as do cells of a primary culture or epithelial cells directly obtained from a tissue sample. It will be understood that the person skilled in the art will select the starting  
30 material depending on the assay. Hence, if a first round assay is to be carried out a cell culture design seems to be most appropriate, while in case for further rounds, i.e. assessing



the activity of potential candidates, the tissue or even the animal model seems to be more appropriate.

The epithelial cells are exposed to a substance of interest for a time period sufficient to ensure a contact of the substance with the cells. In a next step the epithelial cells are exposed to a stress situation, which may be effected e.g. by irradiating the cells with different dosages of UV light, or adding hydrogen peroxide or toxic chemicals to the cell culture. However, the type of stress is not critical as long as the cells are challenged to initiate processes, normally started under stress situations, such as e.g. the production of pro-inflammatory cytokines e.g. IFN- $\gamma$ , TNF- $\alpha$ , IL-1, IL-6, IL-8, apoptosis, altered lipid metabolism, increased production of p53, altered cell signaling as a result of altered patterns of cell surface receptor co-clustering, NF- $\kappa$ B activation, AP1 activation, showing hyperproliferation (anti-apoptosis), altered barrier function etc.. It will be understood that also more than one substance may be tested at the same time, that is a cocktail of one or more substances, which might prove beneficial for the second or further round of assaying.

In a next step the effect of said stress on the epithelial cells is determined by assessing one or more of the following features, for example: epithelial proliferation (PCNA: Ouhtit et al., American Journal of Pathology [2000], 156: 201-207; BrUd: Lu Y-P et al., Cancer Research [1999], 59: 4591-4602 ); epithelial apoptosis (Tunel Assay; modification of protocol outlined by Ouhtit et al., American Journal of Pathology [2000], 156: 201-207); p53 mutation accumulation (Allele-specific polymerase chain reaction [AS-PCR] and single-strand conformation polymorphism [SSCP], Ananthaswamy et al., Nature Medicine [1997], 3: 510-514); production of pro-inflammatory and immuno-modulatory cytokines (e.g. TNF- $\alpha$ , PGE-2, IL-1, IL-6, IL-8, IL-4, IL-10, Platelet Activating Factor, TGF $\beta$ ); markers of inflammation (e.g. COX-2, iNos); and anti-apoptotic transcription factor (including AP-1, NFkappaB) activity by TaqMan Real-time RT-PCR, ELISA, and Immunohistochemistry; qualitative and quantitative assessment of phospholipids, glycosphingolipid and sphingolipid content (Electron-Spray Tandem Mass Spectrometry); analysis of co-clustering patterns of epithelial cell surface receptor molecules including cytokine receptors (e.g. IL-6), molecules of the

TNF-superfamily of receptors (e.g. CD95/APO-1/Fas) and growth regulating receptors (e.g. EGF, Insulin) by fluorescence resonance electron transfer analysis (FRET); markers of ageing, e.g. elastases, collagenases, metalloproteinases, gelatinase, stromelysins, telomerase.

- 5 The results obtained are then compared with a control, which may simply be an assay, wherein the same type of cells are exposed to the same stress conditions with the proviso, that no compound to be assessed for its CD<sub>1d</sub> blocking capacity is provided. As for the animal model a positive control is represented by a CD<sub>1d</sub><sup>-/-</sup> animal, wherein CD<sub>1d</sub> activity is lacking at all.

10

The following examples illustrate the invention in more detail without restricting the same thereto.

### Example 1

#### 15 **Generation of CD<sub>1d</sub> mutant mice**

- Mouse CD<sub>1d</sub> is encoded by two genes, CD<sub>1d1</sub> and CD<sub>1d2</sub>, that share a high degree of nucleotide sequence identity (Bradbury et al., EMBO J., 7 (1988), 3081-3086). The product of the CD<sub>1d1</sub> gene is recognized by all anti-CD<sub>1</sub> antibodies that have been described, whereas  
20 surface expression of the CD<sub>1d2</sub> product has not yet been demonstrated. In addition, the predicted  $\alpha 2$  domain of the CD<sub>1d2</sub> gene product lacks an intra-domain disulfide bond that is found in the  $\alpha 2$  domain of all published classic and non-classic MHC class I molecules (Bradbury, supra). This disulphide bond is thought to be critical for the folding of the antigen-binding groove. Thus, the CD<sub>1d2</sub> gene may not encode a functional antigen-  
25 presenting molecule, and all functions previously attributed to mouse CD<sub>1</sub> may be effected by the product of the CD<sub>1d1</sub> gene. For this reason, it was decided to introduce a targeted mutation into the CD<sub>1d1</sub> gene, while leaving CD<sub>1d2</sub> intact.

- 30 The CD<sub>1d</sub> gene was isolated from a strain 129/Sv phage library with a probe generated by polymerase chain reaction. The targeting construct was prepared using a 2.8 kb Apal fragment containing the 5' region of the CD<sub>1d</sub> gene, a 3.2 kb BamHI-NotI fragment

containing the 3' region of the CD<sub>1d</sub> gene (the NotI site in this fragment comes from the pBluescript vector into which phage DNA was initially subcloned), a neomycin resistance gene (neo), and the pBluescript plasmid (Stratagene). This construct was designed to delete a fragment of about 200 bp from the exon encoding the  $\alpha 2$  domain of CD1d1. The strain  
5 129/Sv-derived embryonic stem (ES) cell line TL1 was transfected with the NotI-linearized targeting vector. G418-resistant colonies were selected and isolated as described in Van Kaer *et al.*, Cell 71 (1992), 1205-1214. Genomic DNA from individual clones was digested with EcoRI and hybridized with a 2.3 kb ClaI-EcoRI probe from the 5' end of the CD1d1 gene. Recombination was confirmed by digestion with KpnI and hybridization with a 700 bp  
10 BamHI-EcoRI probe from the 3' end of the CD<sub>1d1</sub> gene. Chimeric mice were mated with C57BL/6 mice to score for germline transmission, and heterozygous mutant mice were intercrossed to obtain (C57BL/6x129/Sv) F2 homozygous mutants. Mice were typed for their CD1d1 status by genomic southern blotting with the 5' probe. Mutant mice were healthy and bred normally.

15 Because the ES cells and mouse strain used to generate mutant animals differ in their TL status (129/Sv is a TL+ strain and C57BL/6 is a TL- strain) all mice used in this study were genotyped for TL. To type mice for their TL status, tail DNA was digested with BglII and hybridized with a TL-specific probe that detects a polymorphism between strains 129/Sv  
20 (TL+) and C57BL/6 (TL-) (Pontarotti *et al.*, Proc. Natl. Acad. Sci. USA 83 (1986), 1782-1786). This probe was generated by polymerase chain reaction using a set of primers designed on the basis of published sequences (Pontarotti, *supra*):

5'-TATACAGAGCTCCGTAGGAC-3' ; and

25 5'-AGTTGTCTGCAGCCACGAAC-3'.

The CD<sub>1d1</sub> mutant and wild-type mice were housed in a specific-pathogen-free barrier animal facility, accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Animals were used between 12-16 weeks of age at the start of the  
30 experiments. They were housed in filter-protected cages with a 12h light-dark controlled cycle, and provided with autoclaved NIH open formula mouse chow and water *ad libidum*.

The institutional Animal Care and Use Committee approved all procedures. Within each experiment all mice were aged- and sex-matched.

It is underlined that other genetic backgrounds can be used for creating a CD1d mutant mouse, such as Balb/C genetic background.

5

## **Example 2**

### **UV Irradiation of mice**

10 A bank of five Philips TL-40W/12 sunlamps (Philips, The Netherlands) was used to irradiate the mice. These lamps emit a spectrum from 270 to 400 nm; 54% of the irradiation was within the UVB range (280-315 nm) of the solar spectrum, with 45% being in the UVA (315-400nm) region and less than 1% in the UV-C (240-280 nm) range. The irradiance of the five bulbs averaged 10 W/m<sup>2</sup>, as measured by a UVB PMA research radiometer.

15 The dorsal hair of the mice was removed with electric clippers and the mice were placed into a plexiglass box separated into individual compartments by Plexiglas dividers and covered with a wire top which decreased the incident dose by 14%. For each UV-irradiation, the box was placed each time in the same position under the lamps to compensate for the uneven distribution of energy along the length of the bulbs. The mice were exposed once or twice to  
20 an incident dose of 86 mJ/cm<sup>2</sup> UVB from five Philips TL-40W/12 sunlamps. Mice were exposed to a second dose of UVB radiation 96h after the first exposure. All mice were analyzed for signs of skin damage 24, 48, 72 and 96 h after their last UVB exposure.

25 Visually, a clear difference in the degree of skin damage was observed between wild-type and CD1d knockout mice following UVB-irradiation of their shaved dorsums. Whilst clear and significant skin damage (burning, skin lesions) was exhibited by UV-irradiated wild-type mice, no obvious signs of skin damage were detected in UV-irradiated CD1d knockout mice.

**Example 3****Measurement of apoptosis in epidermis**

Apoptotic cell death was detected using the DeadEnd™ Fluorometric TUNEL System (Promega) which measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3' -OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). TdT forms a polymeric tail using the principle of the TUNEL assay. Briefly, formaline fixed paraffin embedded tissue sections on slides were deparaffinized twice in fresh xylene for 5 min at room temperature. They were washed in 100% ethanol for 5 min and then rehydrated sequentially by immersing the sections through graded (100%, 95%, 85%, 70%, 50%) ethanol washes for 3 min. Afterwards, the sections were immersed in 0.85% NaCl for 5min, washed in PBS and then fixed in 4% paraformaldehyde for 15 min followed by two washes in PBS. After removing residual fluid from the sections by tapping, each tissue section was covered with 20µg/ml proteinase K (Sigma) for 8-10 min at room temperature. After proteinase K treatment, tissue sections were rinsed in PBS and then fixed by immersing in 4% paraformaldehyde for 5 min. This was followed by a wash in PBS, removal of residual fluid by tapping and incubation of the sections in equilibrium buffer (Promega) for 5-10 min. After equilibration, the sections were incubated in a humidified chamber with TdT enzyme for 1 h at 37°C. Sections were soaked in stop buffer (SSC; Promega) for 15 min to terminate the reactions and then rinsed in three changes of PBS. After rinsing, sections were stained with propidium iodide solution freshly diluted to 1µg/ml in PBS for 15 min in the dark. They were then washed three times in deionized water for 5 min, and afterwards, excess fluid wiped off the area surrounding the cells. The sections were then immediately examined under a fluorescence microscope.

At 2, 6, 24, 48, 72 and 96h after UV exposure (acute/chronic) a TUNEL Assay (modification of protocol outlined by Ouhtit et al., American Journal of Pathology [2000], 156: 201-207), of the skin taken from CD1d<sup>-/-</sup> and wild-type mice was carried out. The results revealed that epidermal cells within the skin of CD1d<sup>-/-</sup> mice were undergoing a high degree of apoptosis compared to wild-type mice. In contrast, in wild-type skin the epidermal cells were undergoing significantly less apoptosis.

**Example 4****Measurement of Epidermal Hyperplasia**

- 5 Dorsal skin biopsies were fixed overnight in 4% paraformaldehyde and paraffin embedded. Sections were stained with hematoxylin and eosin (H&E) and viewed by light microscopy.

Following UVB exposure,  $CD_{1d}^{-/-}$  mice exhibited significantly reduced epidermal hyperplasia 48h after the last UVB treatment compared to UV-irradiated wild-type mice.

10

**Example 5****Gene profiling**

- 15 In order to elucidate  $CD_{1d}$  function a gene profiling assay comparing wild-type and  $CD_{1d}$  knockout mouse gene expression had been performed.

20 Skin tissue was extracted from 5 individual wild-type and  $CD_{1d}$  knockout mice and extracted separately using Trizol kit (Invitrogen AG, Basel, Switzerland) and then Qiagen RNeasy mini-kits (Basel Switzerland) according to manufacturer instructions with DNase I treatment to remove any genomic DNA contamination. RNA samples were quantified by OD then analyzed via dynamic gel electrophoresis with the Agilent Bioanalyser for intact 28S and 18S rRNA (All 28 / 18 ratio's were between 1.6 and 2.0). Study samples were judged to contain sufficient amounts of high-quality RNA for hybridization to GeneChips. As another quality control measure, prior to hybridization with Affymetrix GeneChips (Affymetrix, Inc., Santa Clara, CA), we confirmed that all samples gave strong signals for pre-selected genes, using Affymetrix test chips (Test chip 5' / 3' ratios were less than 3.0).

- 25 For skin, 10  $\mu$ g total RNA was the starting material for all individual mouse samples. In general, total RNA was converted to biotinylated cRNA, hybridized in the Affymetrix probe array cartridge, stained, and then quantified. First and second strand cDNA synthesis was performed using the SuperScript Choice System (Invitrogen AG, Basel, Switzerland),

according to manufacturer instructions, but using an oligo-dT primer containing a T7 RNA polymerase binding site. Labeled cRNA was prepared with the RNA Transcript Labeling kit (Enzo Biochem Inc., NY). Biotinylated CTP and UTP were used together with unlabeled NTPs in the reaction, and unincorporated nucleotides were removed with Nucleospin columns ( Macherey-Nagel, Düren, Germany ) .

cRNA (20 µg) was fragmented at 94 °C for 35 min in buffer containing 200 mM Tris-acetate pH 8.1, 500mM KOAc, 150 mM MgOAc. Prior to hybridization, fragmented cRNA in hybridization mix ( Buffer containing 100 mM MES, 1M NaCl, 20 mM EDTA, 0.01% Tween 20, 0.5 ng/µl BSA, 0.1 ng/µl herring sperm and Affymetrix controls ), was heated to 95 °C for 5 min, cooled to 45 °C and loaded onto an Affymetrix probe array cartridge. The probe array was incubated for 16 h at 45 °C at constant rotation (60 rpm), then exposed to Affymetrix washing and staining protocol.

This protocol included:

- one wash with non-stringent buffer ( 6X SSPE, 0.01% Tween 20, 0,005% antifoam )
- one wash with stringent buffer ( 100 mM MES, 0.1 M NaCl, 0.01 % tween 20 )
- First stain with 0.01 mg/ml streptavidin-phycoerythrin conjugate (Molecular Probes) in buffer containing 100 mM MES, 1M NaCl, 0.05% Tween 20, 4 mg/ml of BSA.
- one wash with non-stringent buffer ( 6X SSPE, 0.01% Tween 20, 0,005% antifoam )
- Second stain with 3 µg/ml of biotinylated anti-streptavidin + 0.2 mg/ml of IgG in buffer containing 100 mM MES, 1M NaCl, 0.05% Tween 20, 4 mg/ml of BSA.
- Third stain with 0.01 mg/ml streptavidin-phycoerythrin conjugate (Molecular Probes) in buffer containing 100 mM MES, 1M NaCl, 0.05% Tween 20, 4 mg/ml of BSA.
- one wash with non-stringent buffer ( 6X SSPE, 0.01% Tween 20, 0,005% antifoam )

A mathematical method was developed and applied to the raw GeneChip data for the selection of differentially regulated genes. This method moves beyond setting a single fold change cut-off by considering the standard deviation (SD) in the context of absolute expression, or absolute difference intensity (ADI).

The method include the following steps: (A) data processing by the commercially available "MAS5" Affymetrix program (Santa Clara, CA, USA) and rescaling, (B) logarithmic transformation to distribution normality of the rescaled data, (C) multiple hypotheses (one  
5 per gene) analysis of variance (ANOVA) testing, (D) the determination of the robust mean within condition SD (equation 1), within bins of 200 genes ordered by mean ADI levels, to determine a significance limit SD between condition, named the REGExpress function (equation 2 from Genome Biology 2001 2(12): preprint0009.1-0009.31); and (E) subsequent  
10 ranking of genes by the p value of the REGExpress and ANOVA, to help focus at effect importance. The selection is made with the p value resulting from multiple hypotheses (one per gene) ANOVA testing and/or with the p value resulting from REGExpress.

Probe arrays were scanned at 488 nm using an Argon-ion Laser (made for Affymetrix by Agilent). Readings from the quantitative scanning were analyzed with Affymetrix Gene  
15 Expression Analysis Software.

The findings are summarized in the tables I to III below. The fold increase (+) or decrease (-) is the statistically significant relative fold increase or decrease of a gene expressed in CD1d knockout mice compared to the same gene expressed in wild-type mice. It becomes clearly  
20 evident that blocking CD1d upregulates genes controlling hair follicle development, and down-regulates genes involved in inflammation and cancer development.

Table I  
Genes which regulate hair follicle development

25

| Gene Name         | Fold increase/decrease | Mean Wt | Mean CD1d +/- | Biological Function  |
|-------------------|------------------------|---------|---------------|--|
| mu-crystalline    | +27.0                  | 0.922   | 4.251         | thyroid binding protein regulating hair follicle development |
| Patched homolog 2 | +2.763                 | 3.810   | 4.826         | hair follicle development                                    |



**Table II**  
**Genes which regulate inflammation**

| Gene Name                               | Fold increase/decrease | Mean Wt | Mean CD1d +/- | Biological Function   | Disease Association                                     |
|---|------------------------|---------|---------------|---|---|
| TGF beta activated Kinase               | +1.743                 | 3.630   | 4.186         | Signalling molecule of the p38-MAPKinase and the Stress activated protein Kinase (SAPK) pathways. |   |
| Rel-A(NfKappaB)                         | -0.735                 | 7.350   | 7.043         | anti-apoptotic, induction of inflammatory cytokines.  | inflammatory disorders                                  |
| cytochrome beta                         | -0.738                 | 6.829   | 6.525         | superoxide generation   |   |
| Plasminogen activator inhibitor (PAI-1) | - 0.357                | 3.906   | 2.875         | Serine protease inhibitor. Regulates fibrolysis.  | inflammatory disorders                                  |
| MRP14                                   | - 0.202                | 3.943   | 2.344         | Ca <sup>++</sup> dependent regulatory protein in inflammatory responses.                          | acute and chronic inflammatory responses e.g. Psoriasis |
| Mast cell protease                      | - 0.661                | 6.427   | 6.014         | proteolysis and peptidolysis.   | inflammation  |
| P-Selectin                              | - 0.685                | 6.439   | 6.060         | cell adhesion   | inflammation  |
| TFII-1                                  | +1.373                 | 5.707   | 6.024         | Transcription factor which regulates c-Fos activity   |   |
| Interleukin-6                           | - 0.268                | 1.882   | 0.565         | cytokine: multi-functional  | inflammation  |

5

**Table III**  
**Genes which regulate cancer growth/development:**

| Gene Name                               | Fold increase/decrease | Mean Wt | Mean CD1d +/- | Biological Function                          | Disease Association  |
|---|------------------------|---------|---------------|--|--|
| v-Rel (NfKappaB)                        | -0.735                 | 7.350   | 7.043         | oncogenic-transforms cells                   | cancer   |
| Plasminogen activator inhibitor (PAI-1) | - 0.357                | 3.906   | 2.875         | serine protease inhibitor                    | metastatic tumors  |
| P-Selectin                              | - 0.685                | 6.439   | 6.060         | adherence                                    | facilitates tumor metastasis.                              |
| Cathepsin S                             | - 0.697                | 7.545   | 7.184         | cysteine protease                            | malignancy   |
| Proliferin                              | - 0.234                | 1.870   | 0.419         | regulates angiogenesis                       | mouse fibrosarcomas  |
| Interleukin-6                           | - 0.268                | 1.882   | 0.565         |  | secreted by basal cell carcinomas and malignant melanomas. |
| CSF-1 receptor                          | - 0.750                | 7.196   | 6.909         | Growth factor regulating cell proliferation. | Cancer   |

**Example 6**

Evaluation of the inflammatory response induced by a single topical administration of TPA

Phorbol-12-myristate-13-acetate (TPA) provided by Sigma Aldrich (L'Isle d'Abeau Chesnes BP701, 38297 Saint Quentin Fallavier, France) is dissolved in acetone at the dose of 0.01 % (W/V) and 20  $\mu$ l of the solution is applied topically onto the internal face of the right ear of CD<sub>1d</sub><sup>-/-</sup> mice or wild-type mice in order to induce an acute inflammatory response.

The animals are maintained in individual cages with a standard pellet diet in an animal room with a 12-hour light-dark cycle. The facilities provide a filtered air with a temperature of 22 +/- 2 °C and a relative humidity of 55 +/- 10 %.

The inflammatory response is quantified 6 hours, 24 hours and 48 hours after application by measuring the ear oedema using a micrometer (« oditest » provided by Kroeplin GmbH, Postfach 1255 D36372 Schlüchtern, Germany).

The oedema is calculated as follow :

(oedema = ear thickness of the treated group – ear thickness of the acetone group).

The mean value of CD<sub>1d</sub><sup>-/-</sup> group is compared to the mean value of the wild-type group using the Student's t-test.

**Example 7**

Evaluation of the inflammatory response induced by a single topical administration of arachidonic acid

Arachidonic acid (5-8-11-eicosatetraenoic acid) provided by Sigma Aldrich (L'Isle d'Abeau Chesnes BP701, 38297 Saint Quentin Fallavier, France) is dissolved in acetone at the concentration of 140nM and 25  $\mu$ l of the solution is applied topically onto the internal face of the right ear of CD<sub>1d</sub><sup>-/-</sup> mice or wild-type mice in order to induce an acute inflammatory response.

The animals are maintained in individual cages with a standard pellet diet in an animal room with a 12-hour light-dark cycle. The facilities provide a filtered air with a temperature of 22 +/- 2 °C and a relative humidity of 55 +/- 10 %.

- 5 The inflammatory response is quantified 1 hour, 2 hours, and 4 hours after application by measuring the ear oedema using a micrometer (« oditest » provided by Kroeplin Gmbh, Postfach 1255 D36372 Schlüchtern, Germany).

The oedema is calculated as follow :

- 10 (oedema = ear thickness of the treated group – ear thickness of the acetone group).

The mean value of CD<sub>1d</sub><sup>-/-</sup> group is compared to the mean value of the wild-type group using the Student's t-test.

### **Example 8**

- 15 Evaluation of the DTH (delayed-type hypersensitivity) reaction induced by oxazolone

Oxazolone (4-ethoxymethylene-2-phenyl-oxazol-5-one) provided by Sigma Aldrich (L'Isle d'Abeau Chesnes BP701, 38297 Saint Quentin Fallavier, France) is dissolved in acetone at the concentration of 1% (W/V) and 50 µl of the solution is applied once daily for 4 days on the abdominal skin of shaved CD<sub>1d</sub><sup>-/-</sup> mice or shaved wild-type mice.

- 25 4 days later the animals are challenged by a single administration (20µl) onto the internal face of the right ear of oxazolone dissolved in acetone at the dose of 0.3%. The post-challenge response is quantified 24 hours and 48 hours after application by measuring the ear oedema using a micrometer (« oditest » provided by Kroeplin Gmbh, Postfach 1255 D36372 Schlüchtern, Germany).

The oedema is calculated as follow :

(oedema = ear thickness of the treated group – ear thickness of the acetone group).

- 30 The mean value of CD<sub>1d</sub><sup>-/-</sup> group is compared to the mean value of the wild-type group using the Student's t-test.

**Example 9**

Evaluation of skin damages induced by UV irradiation using a solar simulator

- 5 A solar simulator (Oriel 81050) equipped with an UVC filter is used to irradiate  $CD_{1d}^{-/-}$  mice or wild-type mice.

Irradiation : UVB + UVA doses and to be precised

Effect on epidermis : SBC counts, epidermal hyperplasia measurement

- 10 Effect on the dermis: MMP1 and MMP3 expression with immuno-histochemical methods

**Example 10**

Regulation of  $CD_{1d}$  gene transcription by UV radiation and role of  $CD_{1d}$  in regulating UV-induced COX-2 and TNF- $\alpha$  gene transcription

15

**MICE**

Specific pathogen-free male outbred 129/C57BL/6 wild-type and 129/C57BL/6  $CD_{1d}$  knockout mice were obtained from L. Van Kaer, Vanderbilt University Medical Center (Nashville, TN, USA). The animals were maintained in facilities in accordance with current

- 20 Swiss regulations and standards. They were housed in filter-protected cages, and ambient lighting was controlled to provide 12 h light/12 h dark cycles. Autoclaved open-formula mouse chow and water were provided ad libidum. All animal procedures were reviewed and approved by the Institutional Animal care and Use Committee. Within each experiment all the mice were matched for age and sex. The mice were 16 weeks at the start of each
- 25 experiment.

**UV LIGHT SOURCE**

The UVB source was a bank of five Philips TL-40W/12 sunlamps (Philips, The Netherlands).

- 30 These lamps emit a spectrum from 270 to 400 nm; 54% of the irradiation was within the UVB range (280-315 nm) of the solar spectrum, 45% in the UVA (315-400nm) region and less than 1% in the UV-C (240-280 nm) range. The irradiance of the five bulbs averaged 10

W/m<sup>2</sup>, as measured by a UVB PMA research radiometer. Solar simulated light (UVA + UVB) was produced by a 1000W exon UV solar simulator (Solar Light Company, PA, USA) equipped with a WG-320 atmospheric attenuation filter (1mm thick), a visible/infrared band pass blocking filter (UG-5; 5mm thick), and a dichroic mirror to further reduce visible and infrared energy.

#### UV IRRADIATION OF MICE

The dorsal hair of the mice was removed with electric clippers. For mice being exposed to UVB radiation they were placed into a Plexiglass box separated into individual compartments by Plexiglass dividers and covered with a wire top which decreased the incident dose by 14%. For each UVB-irradiation, the box was placed each time in the same position under the lamps to compensate for the uneven distribution of energy along the length of the bulbs. For mice being exposed to solar UV radiation (UVA + UVB) the mice were anaesthetized to immobilize them prior to being exposed to the beam of the solar simulator.

The mice were exposed once to an incident dose of 86, 215 or 430mJ/cm<sup>2</sup> UVB from five Philips TL-40W/12 sunlamps. Mice exposed to solar light (UVA + UVB) were exposed once to an incident dose of 1680 (1 min), 16,800 (10 min) or 33,600mJ/cm<sup>2</sup> (20 min) solar radiation.

#### UV IRRADIATION OF KERATINOCYTE CELL CULTURES

Confluent cultures of keratinocytes grown in sterile 6-well plates (Corning, Netherlands) were submitted to a single dose (5700mJ/cm<sup>2</sup>) of solar UV irradiation. Treatment was performed without plastic lids after having removed medium and replaced it by sterile HBSS. Control cultures were not irradiated. After UV exposure, HBSS was removed and medium put back on cultures. The cells were incubated at 37°C with 5% CO<sub>2</sub> and at various time points thereafter harvested for RNA.

#### IMMUNOSTAINING OF MOUSE TISSUE

Biopsies of wild-type mouse skin were fixed in formaline before being embedded in paraffin.

Cross sections (5µm thick) of paraffin embedded tissues were made, deparaffinized by gentle

heating, de-hydrated and rehydrated using the following procedure: 2 times for 3 min in Xylol, 3 min in Ethanol 100%, 3 min in Ethanol 95%, 3 min in Ethanol 80% and 3 min in PBS 1X. Fresh skin was also embedded in Tissue-Tek (4583, Sakura Finetek, Torrance, USA) and frozen in liquid nitrogen. The sections were then rehydrated in PBS 1X for few minutes. Sections were stained using the anti-mouse CD1d 1H1 primary mAb and developed using the mouse Histostain-plus kit (ZYMED Laboratories Inc., San Francisco, USA).

#### EXTRACTION OF TOTAL RNA FROM SKIN OR CELL LYSATES

Treated and control cultures of human keratinocytes grown in 6-well plates were placed on ice and washed twice using PBS 1X at 4°C. Cell lysate was obtained by scraping the cells in 350µl of lysis buffer RLT (74104, Qiagen AG, Basel, Switzerland) supplemented with 1% of β-mercaptoethanol and by briefly vortexing them. QIAshredder columns (79656, Qiagen AG, Basel, Switzerland) were used to homogenize cell extracts by centrifugation at 13,000xg for 2 min. Total RNA was then prepared using RNeasy kits (74104, Qiagen AG, Basel, Switzerland) according to the manufacturer's protocols. Genomic DNA contamination was removed with on-column DNase digestion using a RNase-free DNase Set (79254, Qiagen AG, Basel, Switzerland). Skin samples of 1cmx1cm were cut into small pieces and homogenized in 1ml of TRIZOL Reagent (15596-026, Invitrogen AG, Basel, Switzerland) using a rotor-stator homogenizer (Polytron, Kinematica, Luzern, Switzerland). The supernatant obtained after centrifugation at 12,000xg was recovered in a fresh tube and incubated for 5min at room temperature. 0.2ml of chloroform was added to the tube, which was vigorously shaken for 15sec and incubated at room temperature for 2-3min. Samples were centrifuged at 12,000xg for 15min at 4°C. The upper aqueous phase was transferred to a fresh tube and total RNA precipitated using 0.5ml of isopropyl alcohol for 10min at room temperature. The RNA pellet obtained by centrifugation at 12,000xg for 10min at 4°C was washed with 1ml of 75% EtOH followed by centrifugation at 7,500xg for 5min at 4°C. The RNA pellet was finally dried at room temperature and dissolved in 40µl of RNase free water by incubating the samples 10min at 55-60°C. Possible DNA contamination was removed with on-column DNase digestion using a RNase-free DNase Set.

SEMI-QUANTITATIVE RT-PCR**A. Reverse transcription-polymerase chain reaction and PCR reaction**

5  $\mu$ g of total RNA were reverse transcribed by oligo-dT priming to first strand cDNA using the Superscript First-Strand Synthesis System for RT-PCR (11904-018, Invitrogen AG, Basel, Switzerland) according to the manufacturer's instructions. PCR of cDNA was performed to either detect specific expression of a single gene (single PCR) or multiple genes (Multiple gene PCR). For single PCR, 48  $\mu$ l of PCR master mix containing 5  $\mu$ l PCR Buffer, 3  $\mu$ l 25mM  $MgCl_2$ , 1  $\mu$ l 10mM dNTPs, 0.5  $\mu$ l 50  $\mu$ M of both sense and anti-sense oligonucleotides, 3  $\mu$ l DMSO, 34.5  $\mu$ l of water and 0.5  $\mu$ l of 5U/ $\mu$ l Taq DNA Polymerase was added to 2  $\mu$ l of cDNA. All the reagents were purchased from Invitrogen (15558-026 and 18427-013, Basel, Switzerland). The number of cycles and the annealing temperature applied to amplify cDNA samples were specific to each gene tested, one cycle consisting of 30s at 94°C, 30s at x°C and 30s at 72°C, each amplification being preceded by 2min at 94°C and finished by 3min at 72°C. Kit MP-70211 (Maxim Biotechnologies, San Francisco, USA) for multiple gene PCR of genes implicated in apoptosis and inflammation were used as instructed. Prior to using each kit, the condition for running multiple reverse and forward primers at the same time to detect multiple genes was determined. The conditions for using kit MP-70211 which contained primers for detecting TNF- $\alpha$  and COX-2 genes, were as follows: 96°C for 1min and 60°C for 4 min (cycles 2x); 94°C for 1min and 60°C for 2 min (cycles 29x); 70°C for 10 min (cycle 1x) and 25°C soak. The DNA sequence of the reverse and forward primers used the MPCR kit for detecting multiple genes under one set of conditions were proprietary and thus are not described in this report.

**Primer Sequences and Number of Cycles of Amplification****Human:**

| Gene  | Primer    | Sequence (5'- 3')               | Annealing temper. | No. of cycles | Product Size |
|-------|-----------|---------------------------------|-------------------|---------------|--------------|
| GAPDH | sense     | AAT CCC ATC ACC ATC TTC CA      | 52                | 16            | 558          |
|       | antisense | GTC ATC ATA TTT GGC AGG TT      |                   |               |              |
| CD1d  | sense     | GCT CAA CCA GGA CAA GTG GAC GAG | 66                | 27            | 452          |
|       | antisense | AGG AAC AGC AAG CAC GCC AGG ACT |                   |               |              |

## Mouse:

| Gene   | Primer    | Sequence (5'- 3')                 | Annealing<br>temper. | No. of<br>cycles | Product<br>Size |
|--------|-----------|-----------------------------------|----------------------|------------------|-----------------|
| GAPDH  | sense     | TTC ACC ACC ATG GAG AAG GC        | 60                   | 22               | 236             |
|        | antisense | GGC ATG GAC TGT GGT CAT GA        |                      |                  |                 |
| CD1d.1 | sense     | ACG TCC TGG CAG ACA GTC CCA GG    | 60                   | 24               | 706             |
|        | antisense | TTA ATG TTG AAA AGA GCG TAC TGG C |                      |                  |                 |

## B. Relative Quantification of mRNA levels

- 5 Amplification of the genes was analyzed by loading 10µl of the PCR products on a 3% agarose gel which was run in a 1XTAE buffer containing 2% Ethidium Bromide at 150V for 30min. The PCR products were visualized as fluorescent bands under UV light. Gels were scanned using a Kodac DC 120 Camera and fluorescence intensity of the bands was quantified using the Software Scion Image β 4.02 Win (Scion Corporation, Maryland, USA).

10

- While studies of CD1d proteins in the murine system suggest a widespread and constitutive expression on many hematopoietic cell types as well as intestinal epithelial cells, and hepatocytes, it was not known whether this molecule is expressed by normal and/or UV exposed mouse skin cells, especially keratinocytes. To address this question, mouse skin from unirradiated and UVB-irradiated wild-type mice was fixed in formaline, sectioned and stained using an anti-mouse CD1d mAb (1H1) to detect CD<sub>1d</sub> protein. Detection of CD<sub>1d</sub> protein in normal unirradiated skin was negative (data not shown). However, CD1d protein was detected (brown color) in the epidermis and dermis of UVB-irradiated mouse skin (Fig. 5). Staining was largely confined to the more differentiated layers of the skin (stratum granulosum and stratum corneum) and at the cellular level was localized to the cytoplasm and nuclear membrane.

20

Thus, UVB-induced mouse skin damage/burning may be directly regulated at the level of the mouse keratinocyte rather than by antigen-presenting cells (locally or systemically).

25

### CD1D GENE TRANSCRIPTION IS REGULATED BY UV RADIATION

Having demonstrated that UVB-induced skin damage is regulated by CD<sub>1d</sub> and that CD<sub>1d</sub>



protein is expressed by mouse epidermal cells (keratinocytes), it was next important to establish whether skin CD1d expression is regulated by UV radiation. Any indication that skin CD1d expression is regulated by UV radiation would suggest that modulation of CD1d levels in skin is a critical factor responsible for regulating UV-induced skin damage. To address this question, the shaved dorsum of wild-type mice was exposed to a single dose (86mJ/cm<sup>2</sup>) of UVB radiation and at various times post irradiation (6, 24, 48, 72 and 96h) the irradiated skin was excised, RNA extracted and purified, and CD1d mRNA levels determined by semi-quantitative RT-PCR. As a control, normal non-irradiated mouse skin was excised, RNA extracted and purified and CD1d mRNA levels determined by semi-quantitative RT-PCR.

As shown in Fig. 6, the level of CD1d mRNA in whole mouse skin which decreased as early as 6h after UVB exposure was significantly reduced 24h post irradiation compared to levels detected in normal non-irradiated skin. In contrast, 48, 72 and 96 hours following UVB exposure CD1d mRNA levels were raised above the levels detected in normal unirradiated control skin. To further validate our studies on the effect of UVB radiation on skin CD1d gene transcription, we exposed the shaved dorsum of wild-type mice to varying doses of solar UV irradiation (UVB +UVA) - 1680 mJ/cm<sup>2</sup> (1 min), 16,800 mJ/cm<sup>2</sup> (10 min) or 33,600 mJ/cm<sup>2</sup> (20 min) of solar UV. At 6 and 72h post irradiation the irradiated skin was excised, RNA extracted and purified, and CD1d mRNA levels determined by semi-quantitative RT-PCR (Fig. 7). As with UVB exposure, we observed a similar decrease and increase in CD1d mRNA levels 6 and 72h following solar UV irradiation, respectively, regardless of the UV dose, suggesting that the response of skin CD1d gene transcription to UV radiation is an important event in the skin's response to the damaging effects of UV exposure.

#### HUMAN KERATINOCYTE CD1D GENE TRANSCRIPTION IS REGULATED BY SOLAR UV RADIATION

In an attempt to address whether human CD1d gene transcription is regulated by UV radiation we investigated whether cultured human keratinocytes exhibit a similar gene transcription

kinetic profile in response to UV irradiation. At different time points following exposure of triplicate DK7 cell keratinocyte cultures to a single dose of 5700mJ/cm<sup>2</sup> solar UV radiation, the cells were harvested for RNA and semi-quantitative RT-PCR performed to determine the relative level of CD<sub>1d</sub> mRNA (Fig. 8). As observed with UV irradiated (UVB or solar) mouse skin, CD<sub>1d</sub> mRNA levels decreased 6h post- irradiation compared to normal non-irradiated controls. Analysis of CD<sub>1d</sub> mRNA levels 10h post-irradiation revealed that these levels had further decreased compared to the levels detected in normal non-irradiated cell cultures. Between 16 and 48h hours after UV exposure, the level of CD<sub>1d</sub> mRNA increased proportionally; a pattern also observed in the skin of UV-irradiated whole wild-type skin suggesting that UV-induced CD<sub>1d</sub> gene transcription in mouse skin was likely being regulated at the level of the keratinocyte.

Human keratinocyte CD<sub>1d</sub> gene transcription is responsive to UV radiation and appears to be regulated in a similar manner to mouse skin CD<sub>1d</sub> implying that a) skin CD<sub>1d</sub> plays a critical role in regulating the response of skin to UV irradiation and b) modulation of CD<sub>1d</sub> levels in skin is a critical factor responsible for regulating UV-induced skin inflammation/damage.

#### GENE TRANSCRIPTION OF KEY GENES WHICH REGULATE SKIN INFLAMMATION IS DIMINISHED IN UVB IRRADIATED CD<sub>1d</sub> KNOCKOUT MOUSE SKIN

To further validate CD<sub>1d</sub> as a critical molecule responsible for regulating UVB-induced skin inflammation/damage we next investigated whether COX-2 and TNF- $\alpha$  gene transcription (key genes responsible for regulating UVB-induced skin inflammation/damage) is deregulated in UV-irradiated CD<sub>1d</sub> knockout mouse skin. It was found that COX-2 and TNF- $\alpha$  mRNA levels in CD<sub>1d</sub> knockout mouse skin were inhibited 48 and 72h after UV irradiation (Fig. 9). Since UV-induced skin damage/inflammation in wild-type mice is observed at 48 and 72h after UV exposure these data demonstrate that skin CD<sub>1d</sub> mediates UV-induced skin inflammation/damage by inducing COX-2 and TNF- $\alpha$  gene transcription.

**Example 11**

Inflammatory cytokines synthesis in UV-irradiated skin of CD<sub>1d</sub> Knockout mice is decreased compared to wild-type control mice.

**5 UV irradiation of mice**

The mice were exposed once to an incident dose of 200 mJ/cm<sup>2</sup> UVB radiation. Three month old female inbred 129/C57BL/6 WT and 129/C57BL/6 CD1d KO mice were involved in this study (n=4).

**10 Cytokine quantification**

8 mm punch biopsies of skin were harvested at 0h, 24h, 48h, 72h, 96h and 168h post-irradiation and immediately frozen in liquid nitrogen. IL-6 and MIP1- alpha were quantified in skin homogenates using classical ELISA methods.

- 15 In WT mice UV-B irradiation induces a high up-regulation of inflammatory cytokines synthesis, 48 hours post-irradiation, whereas in CD<sub>1d</sub> KO mice synthesis of IL-6 and MIP1-alpha protein is significantly reduced. This demonstrates a major role for CD<sub>1d</sub> in UVB-induced cutaneous inflammation.

**Example 12**

Hydrocortisone down-regulates chemical stress-induced CD<sub>1d</sub> gene transcription.

Primary human keratinocytes were grown in complete KGM before being exposed to 300µM H<sub>2</sub>O<sub>2</sub> as a stress factor. 48 hours after seeding, KGM medium was replaced by KGM without hydrocortisone and cultures were treated with 300 µM of H<sub>2</sub>O<sub>2</sub>. Total RNA was extracted at different time after treatment and CD<sub>1d</sub> mRNA quantified by Real Time PCR.

For Taq Man Assays, Applied Biosystems recommends to use 10 to 100ng of initial RNA quantity per well. Consequently, first strand cDNA synthesis was performed in a 20µl volume using 1µg of total RNA and 150µg of random hexamers following the manufacturer's recommendations (Superscript<sup>TM</sup> First-Strand Synthesis System for RT-PCR, 11904-018, Invitrogen). 1µl of the resulting cDNA samples was used for amplification by Real Time PCR.

The sets of primers and probes used for detection of CD1d cDNA were provided by Applied Biosystems as Assays on Demand (respectively Hs00174321\_m1 and Hs00166289\_m1). The primers and probes for the housekeeping gene GAPDH were provided as PDARS (4310884E, Applied Biosystems).

All the cDNA samples were tested in triplicate. PCR reaction mixtures were prepared on ice in micro centrifuge tubes. For one replicate, a pre-mix of 24µl was made using 1.25µl of 20X Target or Control mix, 10.25µl of water and 12.5µl of 2X TaqMan Universal Master Mix, and added to 1µl of cDNA. The PCR reaction mixtures were gently and quickly centrifuged before being aliquoted at the rate of 25µl per well of a 96-wells plate. The plate was sealed, centrifuged at 2000rpm for 30 seconds and placed in a 5700 Sequence Detection System for thermal cycling and fluorescence analysis using the following PCR program:

- 2min at 50°C

- 10min at 95°C

- 40 cycles of 15 sec at 95°C and 1 min at 60°C

Results were analyzed using the GeneAmp® 5700 SDS software. Amplification plots showing amplification of cDNA of interest in function of the number of cycles were obtained.

- 5 The gene expression fold changes obtained in the test condition compared to the control condition were determined by the comparative Ct method using the following formula:

$$\text{Fold changes} = 2^{-\Delta\Delta\text{Ct}} = 2^{-(\Delta\text{Ct test} - \Delta\text{Ct control})}$$

$$\text{where } \Delta\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{housekeeping gene}}$$

As shown in Fig. 11, modulation of CD<sub>1d</sub> gene expression is observed over time when cells are exposed to H<sub>2</sub>O<sub>2</sub>. In presence of Hydrocortisone, the pattern of CD<sub>1d</sub> gene expression obtained following H<sub>2</sub>O<sub>2</sub> challenge differed in that hydrocortisone suppressed CD<sub>1d</sub> transcription.

Thus, Hydrocortisone is able to down-regulate CD<sub>1d</sub> expression in cells subjected to a stress.

### Example 13

Phospholipid levels are disregulated in the skin and the intestine of CD<sub>1d</sub> knockout mice compared to wild-type skin.

Mice: Female inbred C57BL/6 CD1d<sup>-/-</sup> and wild-type C57BL/6 mice aged 5 months were sacrificed and the respective tissue excised. The pieces of skin/intestinal tissue from each mouse were snap frozen using liquid nitrogen. They were then analysed for lipid content.

The main family of lipids regulated by CD<sub>1d</sub> in skin were phospholipids.

|    |                         | nMolar per gram of skin tissue |              |
|----|-------------------------|--------------------------------|--------------|
|    |                         | CD1d -/-*                      | Wild-type    |
| 5  | Spingomyelin            | 33 +/- 30.4                    | 176 +/- 116  |
|    | Lysophosphatidylcholine | 3.8 +/- 3.7                    | 10.0 +/- 4.1 |
| 10 | Phoshatidylcholine      | 35.1 +/-21.1                   | 8.5 +/- 12.9 |
|    | Phosphatidylserine      | 67 +/- 30.4                    | 115 +/- 33   |

15 \*The values are statistically significantly different from wild-type control ( $p < 0.05$ )

### Sphingomyelin

It is a ubiquitous component of animal cell membranes, where it is by far the most abundant sphingolipid. Indeed, it can comprise as much as 50% of the lipids in certain tissues, though  
 20 it is usually less abundant than phosphatidylcholine. For example, it makes up about 10% of the lipids of brain. It is the single most abundant lipid in erythrocytes of most ruminant animals, where it replaces phosphatidylcholine entirely. In this instance, there is known to be a highly active phospholipase A that breaks down the glycerophospholipids, but not sphingomyelin. Like phosphatidylcholine, sphingomyelin tends to be most abundant in the  
 25 plasma membrane, and especially in the outer leaflet, of cells.

Now, it is known that sphingomyelin (and other sphingolipids) and cholesterol may be located together in specific sub-domains ('rafts' or related structures termed 'caveolae') of membranes. As sphingolipids containing long, largely saturated acyl chains, they pack more  
 30 tightly together, thus giving sphingolipids much higher melting temperatures than membrane glycerophospholipids. This tight acyl chain packing is essential for raft lipid organization, since the differential packing facility of sphingolipids and phospholipids is believed to lead to phase separation in the membrane, giving rise to sphingolipid-rich rafts ('liquid-ordered'

phase) surrounded by glycerophospholipid-rich domains ('liquid-disordered' phase). Interactions between specific cellular proteins and lipids in these rafts are believed to be important in signalling mechanisms implicating an important role for sphingomyelin in regulating cell signaling.

5

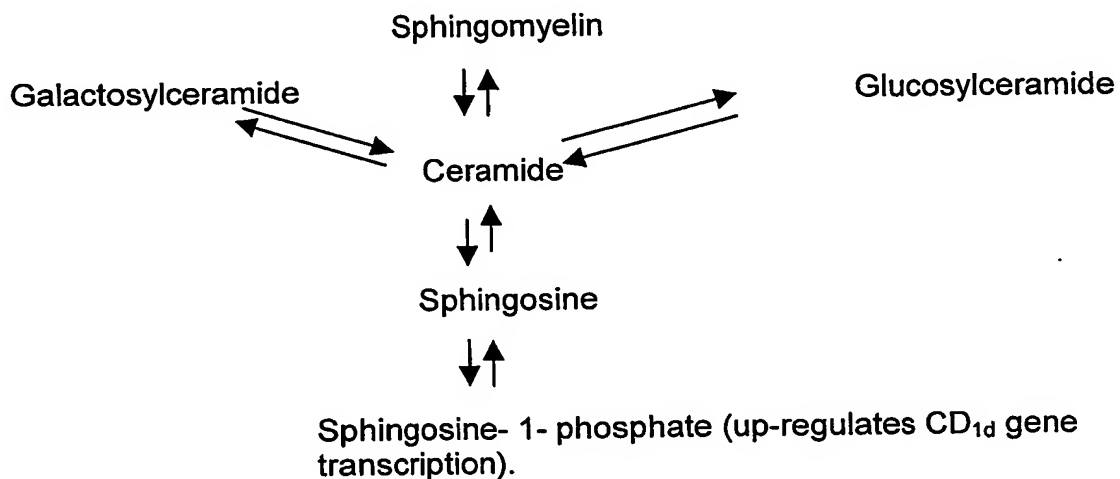
**Sphingomyelin is a key lipid in signal transduction processes involved in apoptosis.**

Also, sphingomyelin serves as a precursor for ceramides, long-chain bases and sphingosine-1-phosphate, as part of the 'sphingomyelin cycle', and many other important sphingolipids. (see figure below). Some of these have functions as intracellular messengers, and others are essential membrane constituents

10

15

20



### Lysophosphatidylcholine

25

- a phospholipid that is pro-inflammatory
- elevated in lesional psoriasis
- intracutaneous injection induces skin inflammation
- formed by the action of **phospholipaseA2** which is the rate limiting step in the production of arachadonic acid. (link to regulation of COX-2 by CD<sub>1d</sub>).

For the large intestine the following results were obtained:

|                               |             |                       | Large Intestine           |             |
|-------------------------------|-------------|-----------------------|---------------------------|-------------|
|                               |             |                       | nMoles per gram of Tissue |             |
| Lipid Class                   | Saturation  | Fatty Acid Family     | CD1d-/*                   | Wild-type   |
| Cardiolipin                   | Saturated   | Stearic Acid          | 405 ± 89                  | 950 ± 442   |
|                               |             | Total                 | 405                       | 950         |
|                               | Unsaturated | Vaccenic Acid         | 74 ± 21                   | 167 ± 45    |
|                               |             | Oleic Acid            | 216 ± 42                  | 377 ± 86    |
|                               |             | α Linolenic Acid      | 23 ± 9                    | 43 ± 9      |
|                               |             | DHA                   | 164 ± 92                  | 596 ± 278   |
|                               |             | Linoleic Acid         | 781 ± 210                 | 2066 ± 727  |
|                               |             | DGLA                  | 36 ± 12                   | 60 ± 16     |
|                               |             | Total                 | 1294                      | 3309        |
| Total Cardiolipin             |             |                       | 1699                      | 4259        |
| Lysophosphatidylcholine       | Saturated   | Myristic Acid         | 78 ± 41                   | 27 ± 5      |
|                               |             | Arachidic Acid        | 7 ± 3                     | 3 ± 0.5     |
|                               |             | Total                 | 85                        | 30          |
|                               | Unsaturated | Eicosenoic Acid       | 9 ± 5                     | 3 ± 2       |
|                               |             | Erucic Acid           | 234 ± 143                 | 17 ± 36     |
|                               |             | Eicosadienoic Acid    | 153 ± 98                  | 30 ± 61     |
|                               |             | DGLA                  | 11 ± 5                    | 4 ± 1       |
|                               |             | Docosadienoic Acid    | 12 ± 8                    | 2 ± 3       |
|                               |             | Total                 | 419                       | 56          |
| Total Lysophosphatidylcholine |             |                       | 504                       | 86          |
| Free Fatty Acids              | Saturated   | Pentadecanoic acid    | 31 ± 10                   | 17 ± 5      |
|                               |             | Total                 | 31                        | 17          |
|                               | Unsaturated | Nervonic acid         | 9 ± 5                     | 2 ± 4       |
|                               |             | Total                 | 9                         | 2           |
| Total Free Fatty Acids        |             |                       | 40                        | 19          |
| Cholesterol Ester             | Unsaturated | Eicosapentaenoic acid | 5 ± 3                     | 1 ± 3       |
|                               |             | Palmitelaidic acid    | 49 ± 27                   | 13 ± 8      |
|                               |             | Total                 | 54                        | 14          |
| Diglyceride                   | Unsaturated | Eicosenoic acid       | 11 ± 8                    | 33 ± 16     |
|                               |             | Total                 | 11                        | 33          |
| Phosphatidylcholine           | Unsaturated | Linoleic acid         | 2551 ± 2321               | 6398 ± 2862 |
|                               |             | Total                 | 2551                      | 6398        |
| Phosphatidylserine            | Unsaturated | Linoleic acid         | 501 ± 105                 | 1102 ± 338  |
|                               |             | Total                 | 501                       | 1102        |

5 \* All values were statistically significantly different from wild-type groups (p < 0.05)



For the small intestine the following results were obtained:

5

|                     |                           |                       | Small Intestine           |           |
|---------------------|---------------------------|-----------------------|---------------------------|-----------|
|                     |                           |                       | nMoles per gram of Tissue |           |
| Lipid Class         | Saturation                | Fatty Acid Family     | CD1d-/-*                  | Wild-type |
| Cardiolipin         | Unsaturated               | Eicosenoic acid       | 12 ± 7                    | 21 ± 3    |
|                     |                           | DHA                   | 178 ± 124                 | 480 ± 121 |
|                     |                           | Total                 | 190                       | 501       |
| Phosphatidylcholine | Saturated                 | Behenic acid          | 13 ± 10                   | 27 ± 10   |
|                     |                           | Total                 | 13                        | 27        |
|                     | Unsaturated               | Mead acid             | 7 ± 4                     | 15 ± 4    |
|                     |                           | Eicosatetraenoic acid | 2 ± 3                     | 7 ± 3     |
|                     |                           | DGLA                  | 154 ± 96                  | 310 ± 115 |
|                     |                           | Docosadienoic acid    | 5 ± 4                     | 10 ± 3    |
|                     |                           | Total                 | 168                       | 342       |
|                     | Total Phosphatidylcholine |                       | 181                       | 369       |

\* All values were statistically significantly different from wild-type groups ( $p < 0.05$ )

10

This above data support a role for CD<sub>1d</sub> in the regulation of phospholipid metabolism which controls inflammatory processes.

### Claims

1. A substance capable to block or modify endogenous CD<sub>1d</sub> function, obtainable by a  
5 process comprising the steps of :
  - (a) exposing epithelial cells to a substance of interest,
  - (b) subjecting the epithelial cells to a stress situation,
  - (c) determining the effect of said stress to said epithelial cells by screening for one  
10 or more of the following assays,
    - (i) epithelial hyperplasia (H&E),
    - (ii) epithelial proliferation (BrUd, PCNA),
    - (iii) epithelial apoptosis (TUNEL),
    - (iv) p53 mutation accumulation,
    - (v) quantitative and qualitative assessment of epithelial lipids,
    - 15 (vi) co-clustering patterns of apoptotic and non-apoptotic cell surface  
receptors,
    - (vii) production of pro-inflammatory cytokines,
    - (viii) production of immuno-modulatory cytokines,
    - (ix) markers of inflammation,
    - 20 (x) anti-apoptotic transcription factors,
    - (xi) markers of ageing,
  - (d) comparing the results obtained with a control.
2. The substance according to claim 1, which is capable of preventing and/or treating  
25 detrimental effects of stress to epithelial cells.
3. The substance according to claim 1, which is capable of preventing or treating hair loss.
4. The substance according to any of the preceding claims, which is a compound reducing  
30 the transcription and/or translation of the CD<sub>1d</sub> gene.

5. The substance according to claim 4, which is a polynucleotide antisense to a sequence comprised by the CD<sub>1d</sub>-gene and/or the CD<sub>1d</sub>-mRNA.
6. The substance according to any of the claims 1 to 4, which is a polynucleotide antisense to a sequence comprised by the glucosylceramide synthase gene and/or the glucosylceramide synthase mRNA.
7. The substance according to any of the claims 1 to 4, which is a polynucleotide sense to a sequence comprised by the sphingomyelinase or ceramide synthase gene and/or the sphingomyelinase or ceramide synthase mRNA.
8. The substance according to any of the claims 1 to 4, which is a polypeptide or peptide, binding to CD<sub>1d</sub> and essentially blocking or modifying CD<sub>1d</sub> function.
9. The substance according to claim 8, wherein the polypeptide is an antibody or the variable part of an antibody.
10. The substance according to any of the claims 1 to 4, which is a lipid.
11. The substance according to claim 10, wherein the lipid is a sphingolipid, glycosphingolipid, phospholipid, ganglioside, sterol, fatty acid, glyceride or phosphatidylinositol phosphate.
12. The substance according to claim 10 and 11 which is derived from plants, microbes or animals, or a phytochemicals, especially a natural or synthetic polyphenols, or ingredients of green tea, and a ginkgolide, vitamin, amino acid or carotenoid.
13. The substance according to claim 8, which is a ceramide or a ligand of a receptor belonging to the TNF-superfamily, in particular CD95/APO-1/Fas.
14. The substance according to any of the preceding claims for the preparation of a carrier for the prevention and/or treatment of the detrimental effects of stress to epithelial cells

and/or hair loss.

15. A composition, containing at least a substance according to any of the preceding claims.

5

16. A composition according to claim 15, which is a food composition, a cosmetic composition or a pharmaceutical composition.

10

17. The composition according to claim 16, which is milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, milk based powders, infant formulae, cereal products, fermented cereal based products, mineral water, chocolate or pet food, or lotions, shampoos, creams, sun-screens, after-sun creams, anti-ageing creams and/or ointments or tablets, liquid, dried oral supplement, wet oral supplement, dry tube-feeding or wet tube-feeding or an anti-cancer drug.

15

18. Use of a substance according to any of the claims 1 to 14 or a composition according to any of the claims 13 to 15 for the prevention and/or treatment of damages in epithelial tissues produced by a stress situation and/or for the prevention and/or treatment of hair loss.

20

19. The use according to claim 18, wherein the stress, situation is a chemical stress, a biological stress or a physical stress.

25

20. The use according to any of the claims 19, wherein the chemical stress is exerted by exposure to oxidants or carcinogens, or wherein the biological stress is exerted by exposure to bacteria, viruses, fungi, lipids derived from surrounding cells and/or microbes, or wherein the physical stress is exerted by exposure to UV-irradiation.

30

21. The use according to any of the claims 18 to 20, wherein the damage is skin burning and/or blistering, cataract formation, epidermal hyperplasia, cancer, inflammation, immune suppression, skin ageing.

22. The use according to any of the claims 18 to 21, wherein the epithelial cells are derived from the skin, gut, eye, lung, prostate, liver, breast, kidney and/or the uterus.

5 23. The use according to claim 21, wherein the cancer is breast cancer, colon cancer, prostate cancer, liver cancer, pancreatic cancer, kidney cancer, non-melanoma and melanoma skin cancers.

10 24. A method for identifying CD<sub>1d</sub> blocking or modifying substances, which comprises the following steps:

- (a) exposing epithelial cells to a substance of interest,
- (b) subjecting the epithelial cells to a stress situation,
- (c) determining the effect of said stress to said epithelial cells by screening for one or more of the following assays,

- 15 (i) epithelial hyperplasia (H&E),
- (ii) epithelial proliferation (BrUd, PCNA),
- (iii) epithelial apoptosis (TUNEL),
- (iv) p53 mutation accumulation,
- (v) quantitative and qualitative assessment of epithelial lipids,
- 20 (vi) co-clustering patterns of apoptotic and non-apoptotic cell surface receptors,
- (vii) production of pro-inflammatory cytokines,
- (viii) production of immuno-modulatory cytokines,
- (ix) markers of inflammation,
- 25 (x) anti-apoptotic transcription factors,
- (xi) markers of ageing,
- (d) comparing the results obtained with a control.

30 25. The method according to claim 24, wherein the stress situation is a chemical stress, a biological stress or a physical stress.

26. The method according to claim 25, wherein the chemical stress is exerted by exposure to oxidants or carcinogens, or wherein the biological stress is exerted by exposure to bacteria, viruses, fungi, lipids derived from surrounding cells and/or microbes, or wherein the physical stress is exerted by exposure to UV-irradiation.
- 5
27. The method according to claim 24 to 26, wherein the pro-inflammatory cytokines are selected from the group consisting of IL-1, TNF- $\alpha$ , PGE-2, IL-6, IFN- $\gamma$  or IL-8.
28. The method according to any of the claims 24 to 26, wherein the immuno-modulatory  
10 cytokines are selected from the group consisting of PAF, IL-10, IL-4 or TGF- $\beta$ .
29. The method according to any of the claims 24 to 26, wherein the lipids are selected from the group consisting of phospholipids, sphingolipids and glycosphingolipids.
- 15 30. The method according to any of the claims 24 to 26, wherein the markers of inflammation include COX-2 and iNos.
31. The method according to any of the claims 24 to 26, wherein the anti-apoptotic transcription factors include AP-1 and NFkappaB.
- 20
32. The method according to any of the claims 24 to 26, wherein the markers of aging include elastases, collagenases, metalloproteinases, gelatinases, stromelysins, telomerase.
- 25 33. Use of a substance according to any of the claims 1 to 14 or a composition according to any of the claims 15 to 17 for decreasing multi-drug resistance of cancers.
34. The use according to claim 33, wherein the cancer is skin, gut or breast cancer.
- 30 35. Use of cells expressing and/or over-expressing CD<sub>1d</sub> in an assay for screening for substances modifying and/or blocking CD<sub>1d</sub> function.

36. Use of  $CD_{1d}^{-/-}$  animals as a test model for determining the activity of substances influencing damages in epithelial tissues produced by a stress situation and/or hair loss.
- 5 37. Use of a substance according to any of the claims 1 to 14 in gene therapy.

Fig. 1A

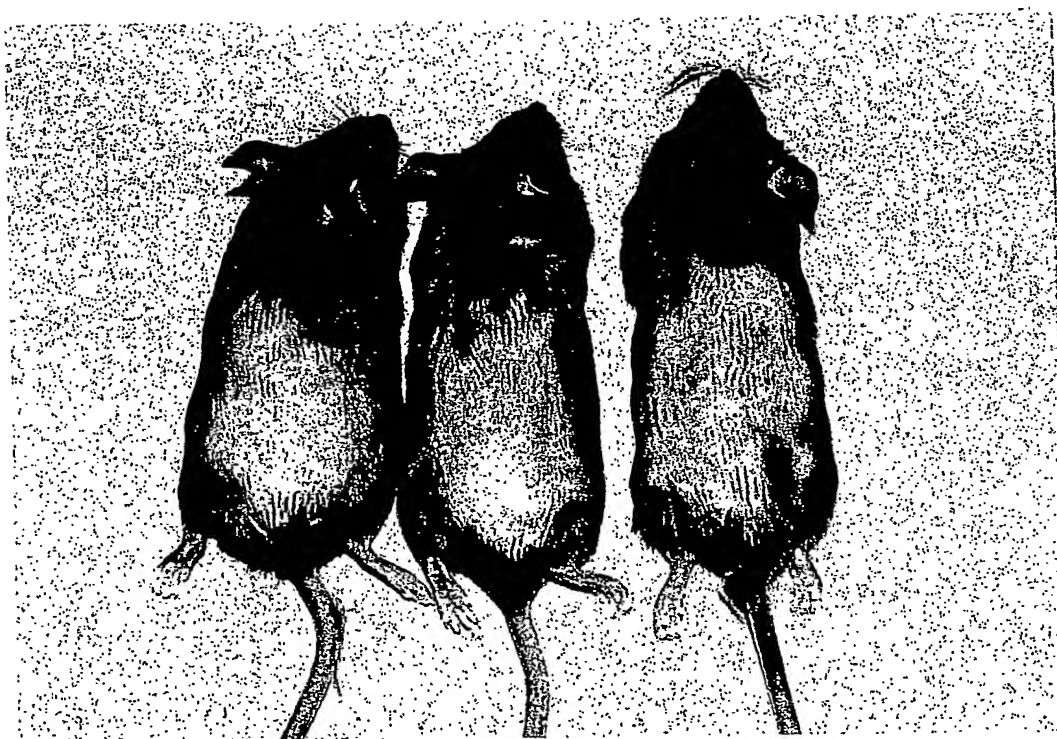




Fig. 1B



Fig. 1C



Fig. 1D



Fig. 2

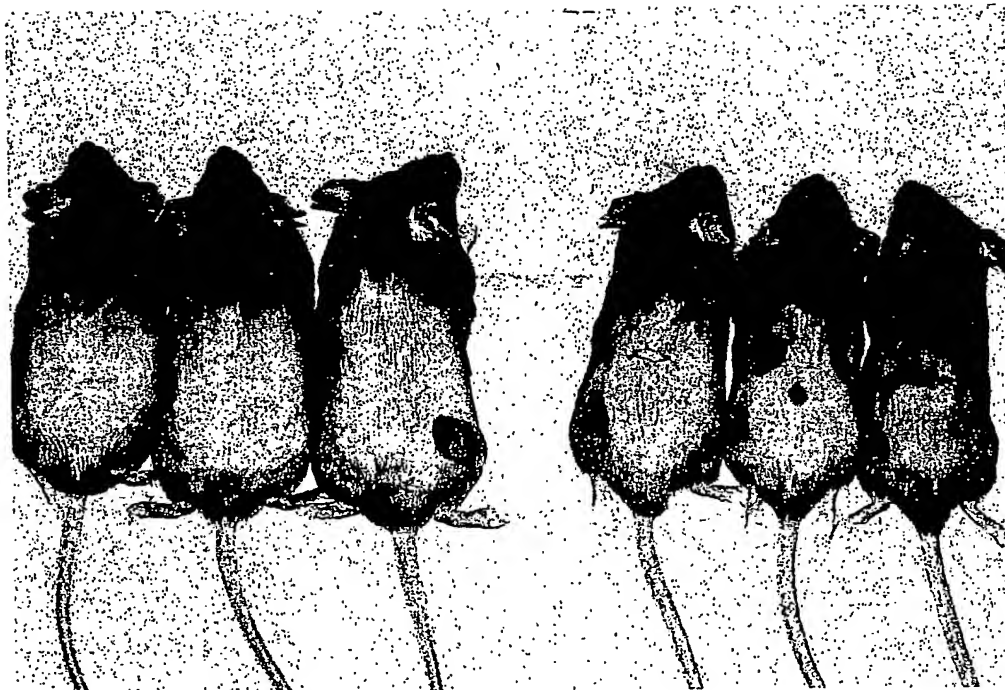
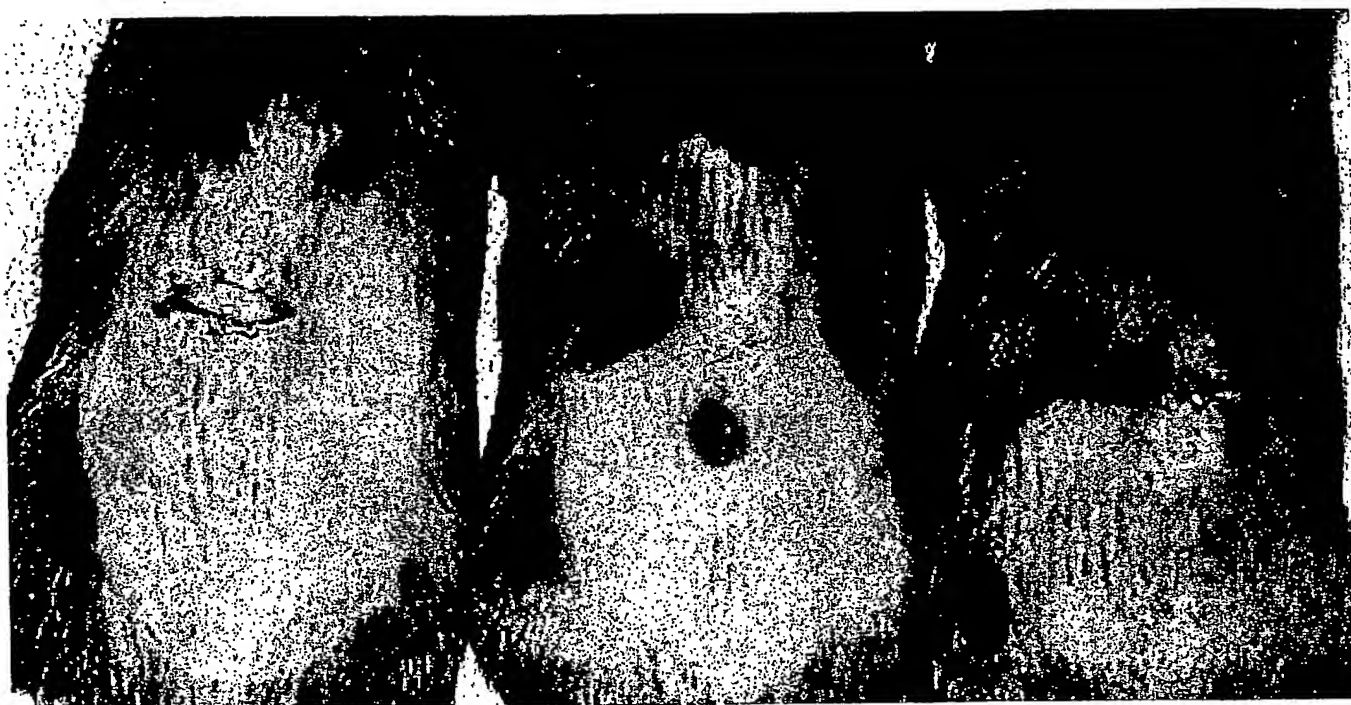


Fig. 2A





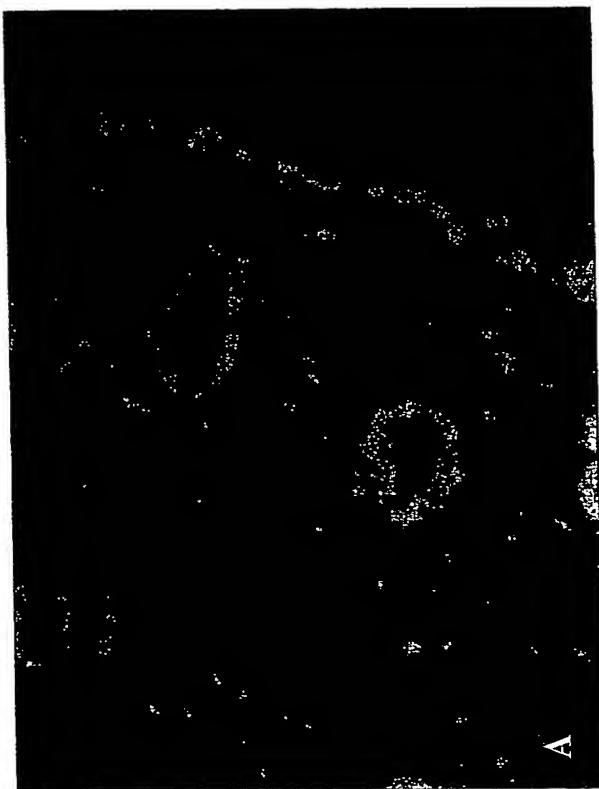
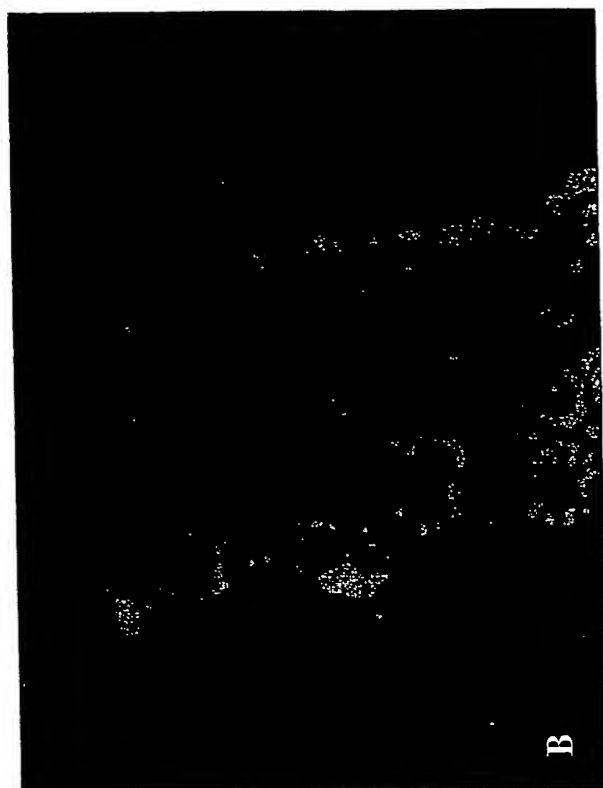




Fig. 4a

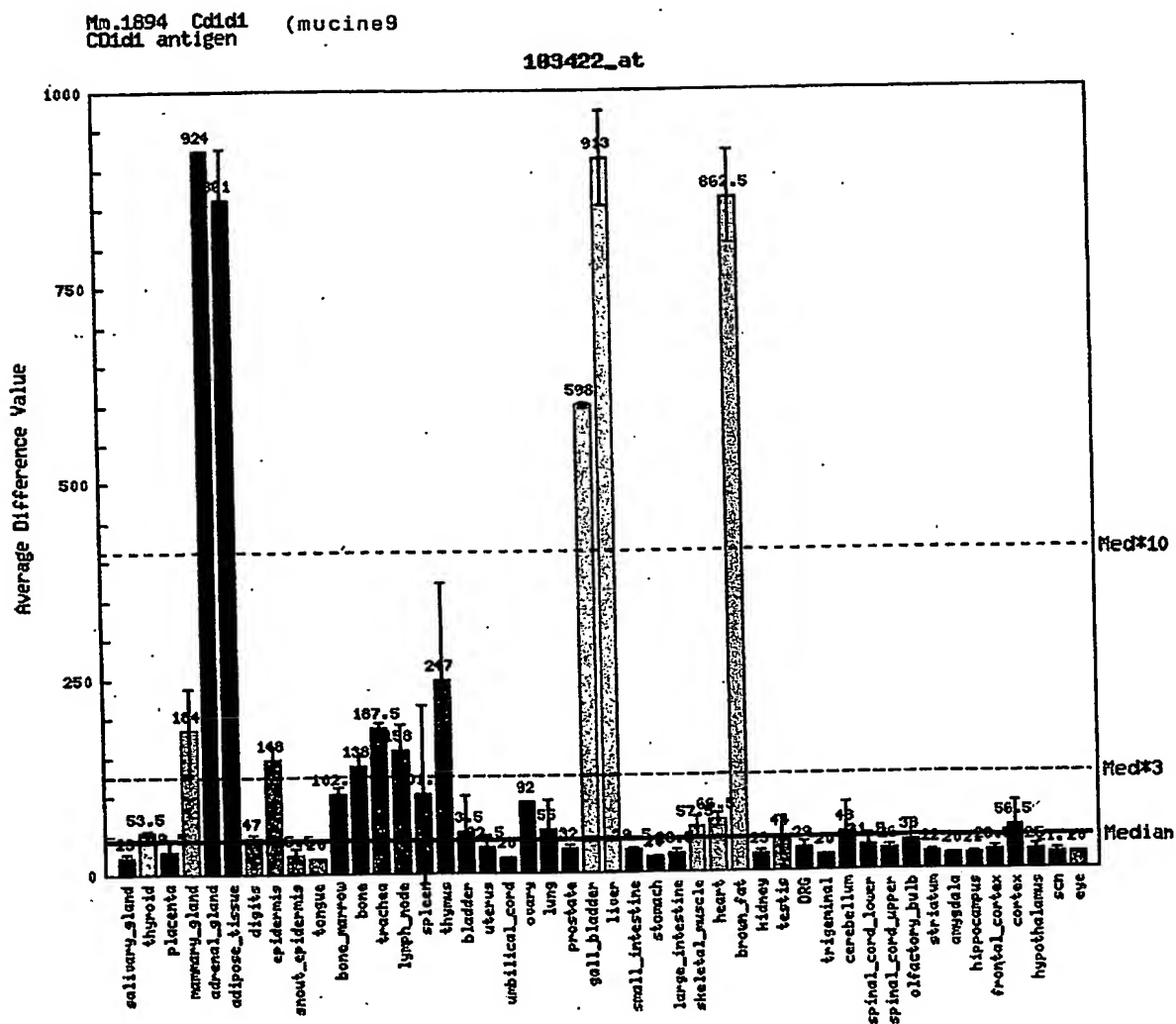
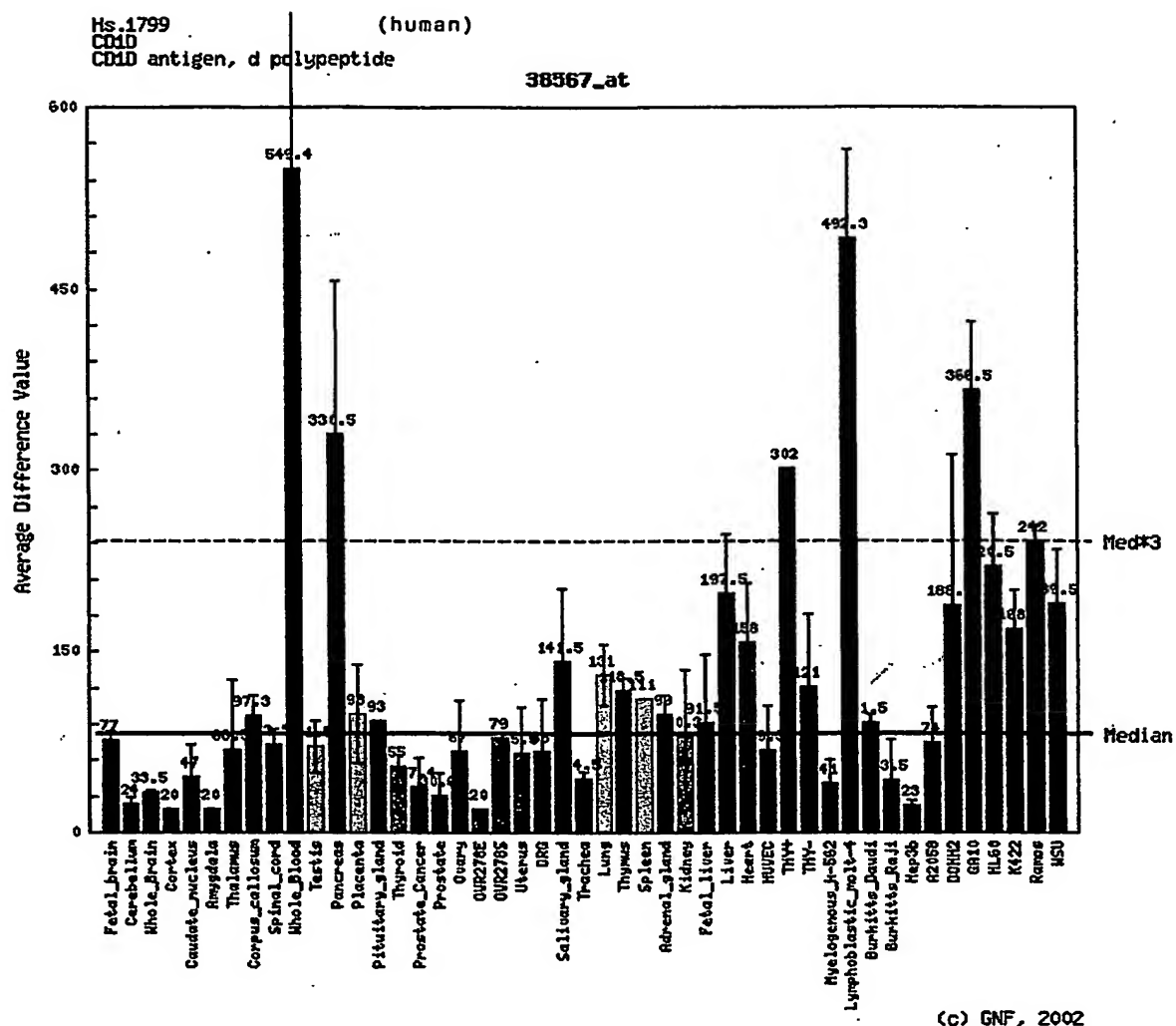




Fig. 4B



# In Silico Promoter Analysis of human and mouse CD1d

Regulation of  
Inflammation

NFkappaB AP1 STAT6

Regulation of Lipid Metabolism

HNF-1 HNF-3 HNF-4 SREBP SF1 PPAR/RXR RARa1 RARa2

[Human CD1D Putative Promoter transcription Binding Sites]

NFkappaB P53

HNF-1 HNF-3

NFY SF1 PPAR/RXR

RARa2

[Mouse CD1D.1 Putative Promoter transcription Binding Sites]

Glucocorticoid receptor

NFkappaB STAT6

HNF-1 HNF-3

NFY SREBP SF1 PPAR/RXR

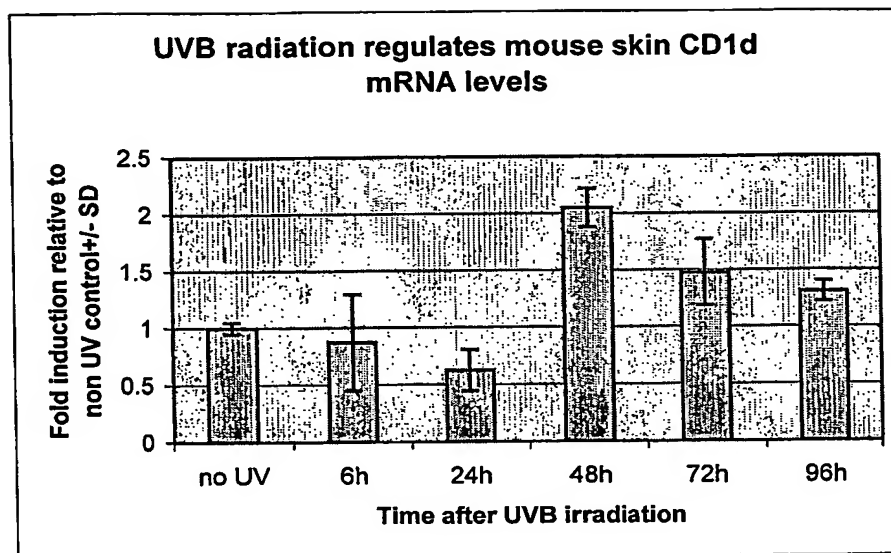
RARa1 RARa2

[Mouse CD1D.2 Putative Promoter transcription Binding Sites]

Fig. 5

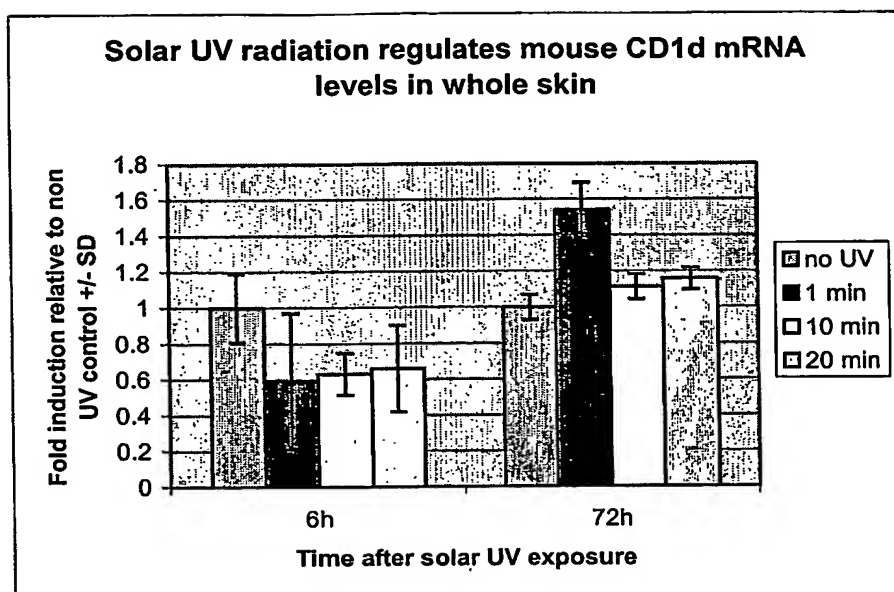


**Figure 5. CD1d protein is expressed in the epidermis of mouse skin 72h following exposure to a single dose (430mJ/cm<sup>2</sup>) of UVB radiation. Formaline fixed mouse skin was stained using an isotype matched control mAb (A) and an anti-CD1d mAb (1H1) (B).**



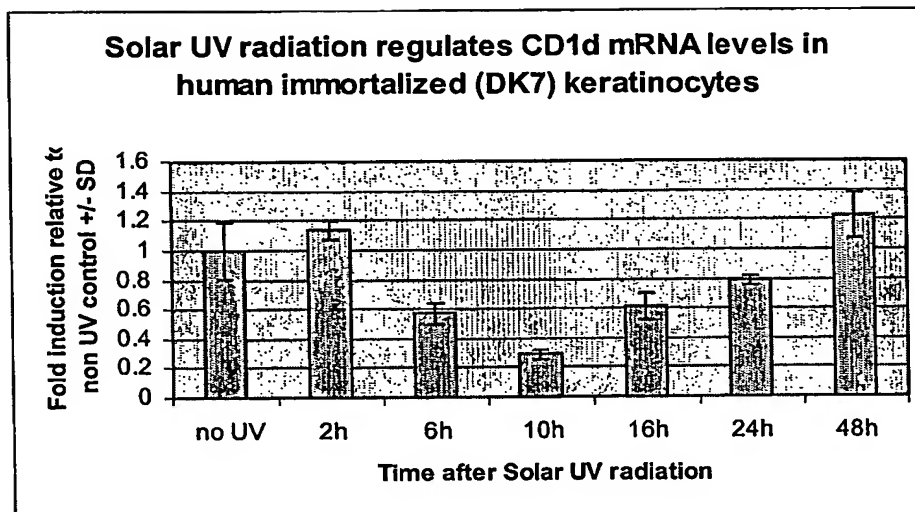
**Fig. 6: Mouse skin CD1d gene transcription is regulated following UVB irradiation.**

The shaved dorsum of wild-type mice (3 mice per experimental group) were exposed to a single dose of  $86\text{mJ}/\text{cm}^2$  of UVB radiation. At different time points (6, 24, 48, 72 and 96h) after UV treatment the mice were sacrificed and their UV exposed skin ( $2 \times 2 \text{ cm}$ ) excised for RNA extraction and purification. Semi-quantitative RT-PCR was then carried out to detect mouse CD1d mRNA. Bars



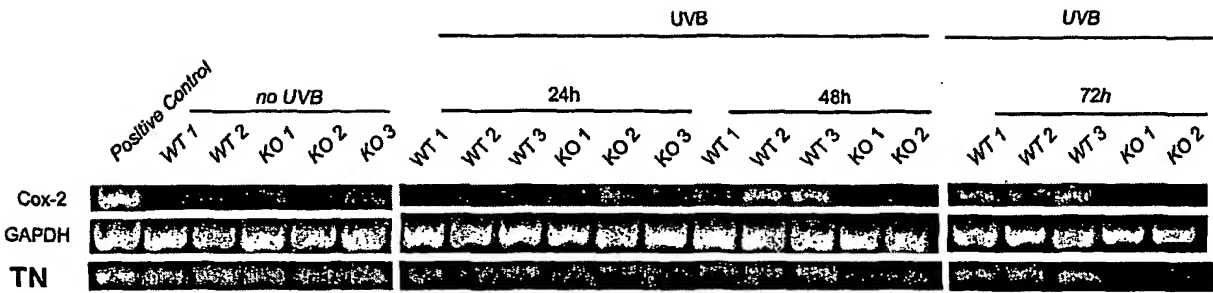
**Figure 7: Mouse skin CD1d gene transcription is regulated following solar simulated light irradiation.**

The shaved dorsum of wild-type mice (3 mice per experimental group) were exposed to different doses -1680 mJ/cm<sup>2</sup> (1 min), 16,800 mJ/cm<sup>2</sup> (10 min) or 33,600 mJ/cm<sup>2</sup> (20 min) of solar UV simulated light. At 6 and 72h



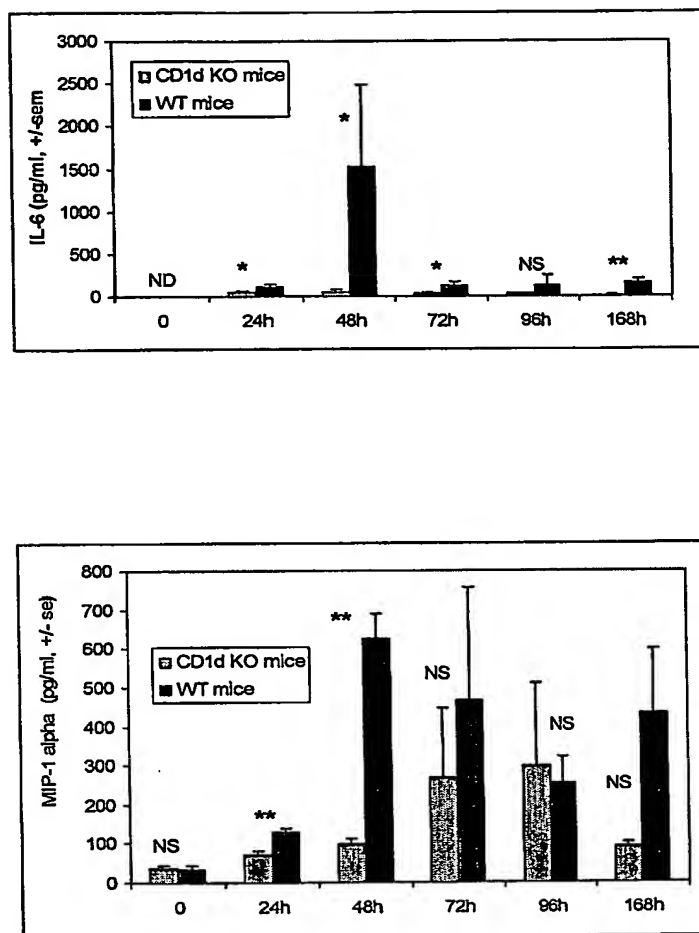
**Fig. 8: CD1d gene transcription in immortalized (DK7) human keratinocytes is regulated following solar UV irradiation**

Medium was removed from DK7 cells grown in triplicate and replaced with sterile HBSS prior to UV exposure. The cells were exposed to 5700mJ/cm<sup>2</sup> of solar UV radiation and immediately after exposure, HBSS was replaced with normal medium. At various time points thereafter (2, 6, 10, 16, 24 and 48h) the cells were harvested for RNA which was then purified and analysed by semi-quantitative RT-PCR to detect human CD1d mRNA. Control cultures were not irradiated. Bars represent the mean intensity fluorescence of gel bands (normalized to GAPDH) relative to non UV exposed control cell cultures +/- SD.



**Fig. 9:** Cox-2 and TNFalpha mRNA levels are down-regulated in UVB-irradiated CD1d knockout mouse skin.

Total RNA was extracted and purified from whole skins excised from wild-type and CD1d knockout mice at various time points (24, 48 and 72h) after exposure to a single dose (86mJ/cm<sup>2</sup>) of UVB radiation. Semi-quantitative RT-PCR was carried out to determine the relative mRNA levels of cox-2



**Fig. 10:** Mouse skin IL-6 and MIP1-alpha are not induced in CD1d KO mice.

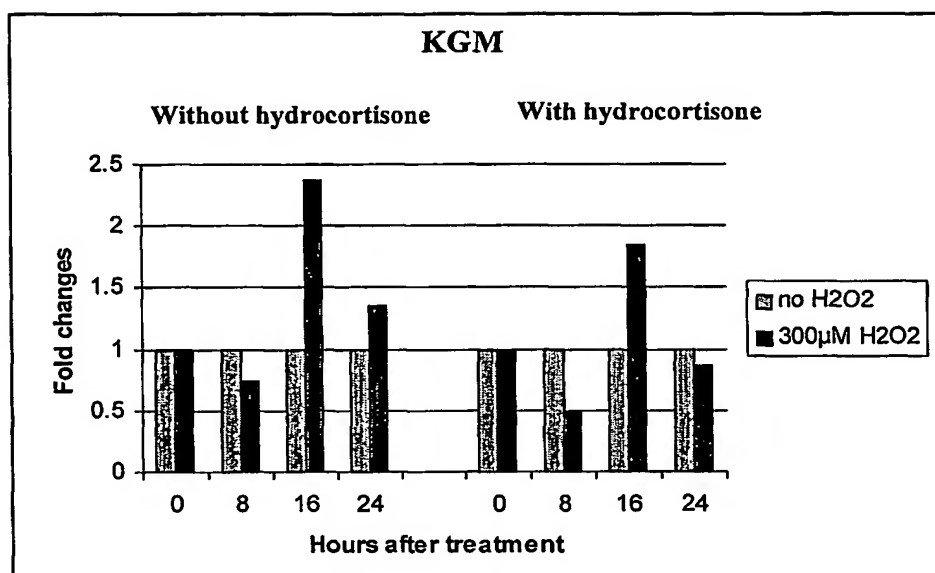
Bars represent the means of 4 animals +/- sem.

Left bar : CD<sub>1d</sub><sup>-/-</sup> mice / right bar : wt mice

Statistical significance were calculated using the Student 't' test; NS, Non-significant, \*  
p ≤ 0.05, \*\*p ≤ 0.01.

ND : non-detectable value.





**Fig. 11.** Effect of H<sub>2</sub>O<sub>2</sub> treatment on CD1d gene expression is lowered by presence of Hydrocortisone.

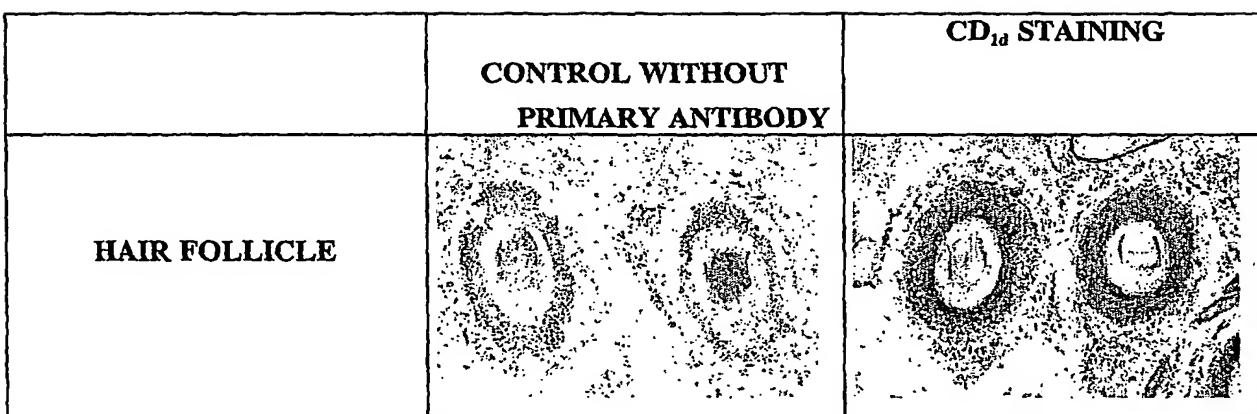


Fig. 12: CD<sub>1d</sub> IS EXPRESSED IN HAIR FOLLICLE OF HUMAN SKIN

# INTERNATIONAL SEARCH REPORT

PCT/03/09496

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC 7 A61K7/48 A61K31/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

WPI Data, PAJ, EPO-Internal, CHEM ABS Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.             |
|------------|--|-----------------------------------|
| P,X        | WO 02 076401 A (GILLESSEN SILKE ;DANA FARBER CANCER INST INC (US); DRANOFF GLENN)<br>( ) 3 October 2002 (2002-10-03)<br>page 7, paragraph 3<br>page 13, paragraph 1<br>page 8, paragraph 1 | 1-4,<br>15-19,21                  |
| X          | WO 00 62787 A (UNIV CALIFORNIA)<br>26 October 2000 (2000-10-26)<br><br>page 2; claims 1,4,5<br>page 11, paragraph 1<br>page 14, paragraph 2<br><br>-/--                                    | 1-4,<br>14-21,<br>24-26,<br>35-37 |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*G\* document member of the same patent family

Date of the actual completion of the International search

29 October 2003

Date of mailing of the International search report

11/11/2003

Name and mailing address of the ISA  
 European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3016

Authorized officer

Sierra Gonzalez, M

## INTERNATIONAL SEARCH REPORT

PCT 03/09496

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No.   |
|------------|---|-------------------------|
| X          | WO 99 22728 A (ARCH DEV CORP ;LIAO SHUTSUNG (US); HIIPAKKA RICHARD A (US))<br>14 May 1999 (1999-05-14)<br>page 6, paragraph 2 -page 7, paragraph 4<br>claims 2-4,7,10<br>---  | 1-4,<br>10-23           |
| X          | WO 01 79152 A (YISSUM RES DEV CO ;DAGAN ARIEH (IL); GATT SHIMON (IL))<br>25 October 2001 (2001-10-25)<br>claims 1-7,13-16<br>---  | 1-4,<br>10-23,<br>33,37 |
| X          | WO 01 12208 A (DRIEU KATY ;SOD CONSEILS RECH APPLIC (FR); UNIV GEORGETOWN (US); P)<br>22 February 2001 (2001-02-22)<br>claim 1<br>---   | 1-14,<br>18-23          |
| X          | K. SHARMA: "Death the Fas way: regulation and pathophysiology of CD95 and its ligand"<br>PHARMACOLOGY AND THERAPEUTICS,<br>vol. 88, 2000, pages 333-347, XP002238439<br>*abstract*<br>---   | 1-14,<br>18-23          |
| A          | KAWANO T ET AL: "CD1d-restricted and TCR-mediated activation of V(alpha)14 NKT cells by glycosylceramides"<br>SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, US,<br>vol. 278, 1997, pages 1626-1629,<br>XP002175772<br>ISSN: 0036-8075<br>the whole document<br>----- | 1-37                    |

# INTERNATIONAL SEARCH REPORT

PCT/EP 03/09496

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1-12, 14-37 (all of them partially)  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-12, 14-37 (all of them partially)

Present claims 1-12 and 14-37 relate to an extremely large number of possible compounds: the property of being capable of block or modify endogenous CD1d function is a property of many compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds mentioned in claim 13.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

PCT/03/09496

| Patent document<br>cited in search report |   | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|---|---------------------|----------------------------|---------------------|
| WO 02076401                               | A | 03-10-2002          | WO 02076401 A2             | 03-10-2002          |
|   |   |                     | US 2002165170 A1           | 07-11-2002          |
| WO 0062787                                | A | 26-10-2000          | AU 4642600 A               | 02-11-2000          |
|   |   |                     | WO 0062787 A1              | 26-10-2000          |
| WO 9922728                                | A | 14-05-1999          | AU 1289899 A               | 24-05-1999          |
|   |   |                     | EP 1027045 A1              | 16-08-2000          |
|   |   |                     | JP 2003524577 T            | 19-08-2003          |
|   |   |                     | WO 9922728 A1              | 14-05-1999          |
|   |   |                     | US 2003105030 A1           | 05-06-2003          |
|   |   |                     | US 2003153541 A1           | 14-08-2003          |
|   |   |                     | US 2003144346 A1           | 31-07-2003          |
|   |   |                     | US 6576660 B1              | 10-06-2003          |
| WO 0179152                                | A | 25-10-2001          | WO 03027058 A1             | 03-04-2003          |
|   |   |                     | AU 5250601 A               | 30-10-2001          |
|   |   |                     | WO 0179152 A1              | 25-10-2001          |
|   |   |                     | US 2003133904 A1           | 17-07-2003          |
| WO 0112208                                | A | 22-02-2001          | AU 6768900 A               | 13-03-2001          |
|   |   |                     | CA 2378052 A1              | 22-02-2001          |
|   |   |                     | CN 1361697 T               | 31-07-2002          |
|   |   |                     | EP 1200108 A1              | 02-05-2002          |
|   |   |                     | JP 2003507336 T            | 25-02-2003          |
|   |   |                     | NO 20020666 A              | 11-02-2002          |
|   |   |                     | WO 0112208 A1              | 22-02-2001          |

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**